Reefolding, Mutagenesis and Characterisation of Secretory Phospholipase A₂

A thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

Amrik Basran
Department of Biochemistry, University of Leicester
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ABSTRACT

Bovine pancreatic phospholipase A\(_2\) (bPLA\(_2\)) is a 14 kDa (123 amino acids) enzyme with seven disulphide bonds. Its primary function \textit{in vivo} is the hydrolysis of dietary phospholipids.

A T7 RNA polymerase based expression system was used to overexpress a synthetic gene encoding bovine pancreatic pro-phospholipase A\(_2\) in \textit{E. coli}. The expressed protein was directed to the periplasmic space via an outer membrane secretory signal (OmpT) where, after translocation the protein formed insoluble inclusion bodies. Translocation efficiency was significantly increased (from about 25\% to over 90\%) when protein expression was induced after the cells had reached the stationary phase of growth (O.D\(_{600}\) ~ 2.8).

Optimal conditions for refolding of bPLA\(_2\) were found to be by rapid dilution under anaerobic conditions, in the presence of 2 mM oxidised glutathione, 4 mM reduced glutathione, 5 mM potassium EDTA and 25 mM sodium borate pH 8.7, with a final protein concentration of approximately 45 mg/L. The final amount of active recombinant protein produced was 22 mg per litre of bacterial culture.

Site directed mutagenesis was used to investigate the role of the 58-71 surface loop of bovine pancreatic PLA\(_2\) in interfacial binding to a variety of aggregated phospholipids and surfactants. The surface loop was mutated so that the amino acid sequence was similar to that found in porcine pancreatic PLA\(_2\). Three mutants were made, Val-63→Phe-63 (V63F), Asn-71→Glu-71 (N71E) and finally the double mutant (V63F/N71E). The mutants were overexpressed in \textit{E. coli} and refolded \textit{in vitro}. V63F had an increased affinity for lipid-water interfaces so that its binding to zwitterionic micelles was more like that of the porcine enzyme but catalysis was still similar to the bovine enzyme. Both N71E and V63F/N71E showed a reduction in binding to zwitterionic micellar interfaces at pH 8 compared with bPLA\(_2\). The affinity for the lipid-water interface could be restored by the addition of a negative charge (via an anionic detergent) to the micellar interface.
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Finally I would like to thank my family for their constant support and encouragement.
ABBREVIATIONS

AMINO ACIDS

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<tr>
<td>Ala</td>
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<td>Asp</td>
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<td>Cys</td>
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<td>Guanine</td>
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<td>T</td>
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DNA  Deoxyribonucleic acid
dNTP  deoxynucleoside triphosphate
ddNTP  dideoxynucleoside triphosphate

ENZYMES

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<tr>
<td>bPLA₂</td>
<td>Bovine pancreatic Phospholipase A₂</td>
</tr>
<tr>
<td>pPLA₂</td>
<td>Porcine pancreatic Phospholipase A₂</td>
</tr>
<tr>
<td>pbPLA₂</td>
<td>Bovine pancreatic Phospholipase A₂ pro-enzyme</td>
</tr>
<tr>
<td>OmpT-pbPLA₂</td>
<td>Bovine pancreatic Phospholipase A₂ pro-enzyme with a outer membrane protein secretory signal sequence</td>
</tr>
<tr>
<td>sPLA₂</td>
<td>Human secretory PLA₂</td>
</tr>
<tr>
<td>psPLA₂</td>
<td>Pro-Human secretory PLA₂</td>
</tr>
<tr>
<td>OmpT-sPLA₂</td>
<td>OmpT-Human secretory PLA₂</td>
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V63F  bPLA2 with the valine-63→phenylalanine mutation
N71E  bPLA2 with the asparagine-71→glutamic acid mutation
V63FN71E bPLA2 with the valine-63→phenylalanine and asparagine-71→glutamic acid mutation

SURFACTANTS

diCgPC  1,2-dihexanoyl-sn-glycero-3-phosphocholine
diCgPC  1,2-dioctanoyl-sn-glycero-3-phosphocholine
HEPC  2-hexadecanoylthio-1-ethylphosphorylcholine
C14PN  n-hexadecylphosphocholine
dIMPE  1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine
dIMPC  1,2-dimyristoyl-sn-glycero-3-phosphocholine
dIMPG  1,2-dimyristoyl-sn-glycero-3-phosphoglycerol
dIMPM  1,2-dimyristoyl-sn-glycero-3-phosphomethanol

GENERAL

2D-HSQC  2D-Heteronuclear single quantum correlation
ANS  8-anilino-1-naphthalenesulphonic acid
APS  Ammonium persulphate
cmc  Critical micellar concentration
DMSO  Dimethyl sulfoxide
DTNB  5,5′-dithionitrobenzoic acid
EDTA  Ethylenediamine tetra-acetic acid
FAF-BSA  Fatty acid free bovine serum albumin
IPTG  Isopropyl β-D-thiogalactopyranoside
Nmr  Nuclear magnetic resonance
NTSB  2-Nitro-5-sulphobenzoate
PAGE  Polyacrylamide gel electrophoresis
PCR  Polymerase chain reaction
PMSF  Phenyl methylsulphonyl fluoride
SDS  Sodium dodecyl sulphate
TEMED  N,N′,N′,N′-tetramethylethylenediamine
U.V.  Ultraviolet
X-gal  5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
CHAPTER 1

INTRODUCTION

1.1 Introduction. 1
1.2 Primary structure classification of extracellular PLA\textsubscript{2}s. 4
1.3 The structure of the pancreatic PLA\textsubscript{2}s. 7
   1.3.1 The active site. 9
   1.3.2 The amino-terminal helix (residues 1-12). 10
   1.3.3 The calcium binding loop (residues 25-42). 12
   1.3.4 Helices C (residues 37-54) and E (residues 90-109). 14
   1.3.5 The Group I loop (58-71). 14
   1.3.6 The \( \beta \)-wing (74-84). 15
   1.3.7 The interfacial recognition site. 16
1.4 Human synovial fluid PLA\textsubscript{2}. 18
1.5 The catalytic mechanism of PLA\textsubscript{2}. 21
1.6 The organisation of aggregated phospholipids. 23
1.7 Enzyme activation at the lipid-water interface. 25
   1.7.1 The interfacial recognition site (IRS) model. 26
   1.7.2 The substrate theory. 30
   1.7.3 The concentration effect. 32
1.8 Kinetic characterisation of PLA\textsubscript{2}s. 32
1.9 Substrate specificity of PLA\textsubscript{2}. 36
1.10 Site directed mutagenesis of PLA\textsubscript{2}. 36
1.11 Aims of the project. 42
CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and reagents.

2.2 Molecular biology.
   2.2.1 PCR mutagenesis.
   2.2.2 PCR screening for gene insertion into the pET12a vector.
   2.2.3 Single base 35S dideoxynucleotide termination sequencing.
   2.2.4 35S dideoxynucleotide termination sequencing.

2.3 Production of pro-sPLA\(_2\) for expression in MEL cells.
   2.3.1 Construction of the pro-sPLA\(_2\) gene.
   2.3.2 Transformation of MEL cells by electroporation.

2.4 Protein expression and purification.
   2.4.1 Overexpression of bovine pro-PLA\(_2\) from E. coli.
   2.4.2 Isolation of pbPLA\(_2\) inclusion bodies from E. coli.
   2.4.3 Overexpression of pro-sPLA\(_2\) in E. coli.
   2.4.4 Isolation of psPLA\(_2\) inclusion bodies from E. coli.

2.5 In vitro refolding and purification of recombinant PLA\(_2\).
   2.5.1 Refolding of pro-bPLA\(_2\) in the absence of molecular oxygen.
   2.5.2 Activation and purification of pro-bPLA\(_2\).
   2.5.3 Purification of bPLA\(_2\) using hydrophobic interaction chromatography.
   2.5.4 Purification of bPLA\(_2\) using ion exchange chromatography.
   2.5.5 Purification of recombinant bPLA\(_2\) by chromatofocusing.
   2.5.6 In vitro protein refolding of pro-sPLA\(_2\).
   2.5.7 Refolding psPLA\(_2\) in the absence of molecular oxygen.
   2.5.8 Activation and purification of psPLA\(_2\).

2.6 Kinetic methods and binding assays.
   2.6.1 Protein concentration determination.
   2.6.2 Cmc determination using ANS fluorescence.
   2.6.3 Fluorescence binding studies of PLA\(_2\) to lysophospholipid analogues.
   2.6.4 Kinetic studies of PLA\(_2\) using the pH-stat.
   2.6.5 Assays with egg-yolk lecithin/deoxycholate mixed micelles.
   2.6.6 \(K_{\text{m}}^{\text{app}}\) determination for micelles of diC\(_4\)PC.
   2.6.7 \(K_{\text{m}}\) determination for monomeric diC\(_4\)PC.
2.6.8 Kinetics on small sonicated vesicles of DMPM. 72
2.6.9 Mixed micelles of diC14PC and sodium deoxycholate. 72
2.6.10 U.V. spectrophotometric assay using micellar HEPC. 73
2.6.11 Labelling of E. coli membranes with (1-14C)-oleic acid. 74
2.6.12 E. coli membrane assay. 75
2.7 Nmr spectroscopy and electrospray mass spectrometry. 75
2.7.1 1D 1H nmr of bPLA2 with the titration of RLM-1. 75
2.7.2 Nmr spectroscopy of the bPLA2 mutant V63F. 76
2.7.3 Electrospray mass spectrometry. 76

CHAPTER 3
OVEREXPRESSION, REFOLDING AND PURIFICATION OF HUMAN SECRETORY PLA2.

RESULTS

3.1 Expression and refolding of pro-sPLA2 from E. coli. 77
3.2 Expression of sPLA2 from MEL cells. 87
    3.2.1 Expression of sPLA2 (construct 1) from MEL cells. 90
    3.2.2 Construction of pro-sPLA2 (construct 2) for MEL cell expression. 92
3.3 Expression of paPLA2 from methylotrophic yeast. 94
3.4 Secretion of sPLA2 into the periplasmic space of E. coli. 96

DISCUSSION

3.5 E. coli derived recombinant sPLA2. 98
    3.5.1 OmpT-sPLA2. 100
3.6 Expression from eukaryotic cells. 103
CHAPTER 4
OVEREXPRESSION, REFOLDING AND PURIFICATION OF BOVINE PANCREATIC PHOSPHOLIPASE A2 FROM E. coli.

RESULTS

4.1 Overexpression of recombinant $^{15}$N-pro-ovine pancreatic PLA$_2$. 106
4.2 Isolation and $S$-sulphonation of $^{15}$N-pbPLA$_2$. 109
4.3 In vitro refolding of $^{15}$N-pbPLA$_2$. 112
4.4 Isolation and purification of $^{15}$N-pbPLA$_2$. 114
  4.4.1 Activation and purification of $15$N-pbPLA$_2$. 115
  4.4.2 Trypsin activation of $^{15}$N-pbPLA$_2$. 116
  4.4.3 Purification of activated $^{15}$N-bPLA$_2$ by hydrophobic interaction chromatography. 116
  4.4.4 Cation exchange chromatography purification of $^{15}$N-bPLA$_2$. 118
  4.4.5 Chromatofocusing of $^{15}$N-bPLA$_2$. 119
4.5 Nmr of recombinant bPLA$_2$ with RLM-1 121
4.6 Purification table of bPLA$_2$. 127

DISCUSSION

4.7 Overexpression of recombinant proteins in E. coli. 128
4.8 In vitro refolding of recombinant PLA$_2$. 135

CHAPTER 5
CHARACTERISATION OF BPLA$_2$ MUTANTS OVEREXPRESSED IN E. coli AND REFOLDED IN VITRO.

5.1 Background. 143

RESULTS AND DISCUSSION

5.2 Molecular biology and mutagenesis of bPLA$_2$. 147
5.3 Mutant protein expression, refolding and purification. 149
5.4 Protein concentration determination using the bichinchoninic acid assay. 152
5.5 Electrospray mass spectrometry. 153
CHAPTER 6

KINETIC CHARACTERISATION OF BOVINE PANCREATIC PLA₂ MUTANTS.

RESULTS

6.1 Determination of critical micelle concentrations of substrates. 160
6.2 Binding of PLA₂ to micelles of C₁₄PN measured by fluorescence. 162
6.3 The egg yolk lecithin/deoxycholate mixed micelle assay. 165
6.4 $K_m^{PPP}$ and $k_{cat}$ determination using diC₄PC micelles. 167
6.5 $k_{cat}$ determination using the diC₄PC monomer assay. 169
6.6 Kinetic characterisation on long-chain phospholipids. 170
6.7 Kinetic characterisation using a spectrophotometric assay. 175

DISCUSSION

6.8 HEPC spectrophotometric microtitre-plate assay. 180
6.9 V63F bPLA₂. 182
6.10 N71E bovine PLA₂. 193
6.11 V63FN71E bovine PLA₂. 200
6.12 An engineered secondary calcium binding site? 203

CHAPTER 7

SUMMARY AND CONCLUSIONS

7.1 Maintaining solubility of recombinant proteins in E. coli. 207
  7.1.1 Expression as a fusion protein. 207
  7.1.2 Co-expression of "helper" proteins. 208
  7.1.3 Disulphide bond formation. 209
7.2 Overexpression of recombinant PLA₂. 210
7.3 In vitro refolding. 211
7.4 The S8-71 surface loop of bPLA₂. 213
APPENDIX A

GENERAL METHODS

A1 Microbiology culture methods. 218
   A1.1 Media. 218
   A1.2 Agar plates. 218
   A1.3 Culture Media. 219

A2 DNA modification reactions. 220
   A2.1 PCR conditions. 220
   A2.2 Digestion of DNA with restriction enzymes. 220
   A2.3 Alkaline phosphatase treatment of DNA. 221
   A2.4 5' phosphorylation of DNA using T4 polynucleotide kinase. 221
   A2.5 DNA ligations using T4 DNA ligase. 221

A3 Molecular biology techniques. 222
   A3.1 DNA gel buffers. 222
   A3.2 Agarose gel electrophoresis. 222
   A3.3 Lambda phage DNA cut with Hind III. 223
   A3.4 Isolation of DNA from agarose gels. 224
   A3.5 TE-saturated phenol/chloroform. 224
   A3.6 TE-Phenol/chloroform extraction of DNA. 224
   A3.7 Isopropanol precipitation of DNA. 225
   A3.8 Plasmid mini preps. 225
   A3.9 Determination of DNA concentration by absorbance spectroscopy. 226
   A3.10 Preparation of competent E. coli cells. 226
   A3.11 Transformation of competent cells. 227
   A3.12 Preparation of glycerol stocks. 227

A4 DNA sequencing. 227
   A4.1 Denaturing double-stranded plasmid DNA and primer annealing. 227
   A4.2 Dideoxynucleotide sequencing. 228

A5 Miscellaneous 229
   A5.1 Synthesis of disodium 2-nitro-5-thiosulphobenzoate (NTSB). 229
   A5.2 Oxidation of reduced glutathione. 229
   A5.3 SDS-PAGE gels. 230
   A5.4 Tris-Tricine SDS PAGE. 230

REFERENCES 232
CHAPTER 1

INTRODUCTION

1.1 Introduction

Phospholipase A2 (PLA2) is an enzyme that catalyses the hydrolysis of the \( sn-2 \) ester bond found in phospholipids (Fig. 1.1). The products of this hydrolysis are a free fatty acid and a lysophospholipid. PLA2 is able to hydrolyse a wide range of phospholipids with a variety of functional head-groups (e.g. choline and glycerol) and acyl carbon chain lengths.

Fig. 1.1: Site of action of PLA2 on phosphatidylcholine. The ester hydrolysis at the \( sn-2 \) position of the phospholipid produces a lysophospholipid and a free fatty acid.

PLA2s are ubiquitously distributed in both prokaryotes and eukaryotes but can be subdivided into two main groups: low and high molecular weight enzymes. The low molecular weight PLA2s are extracellular secreted enzymes whose functions range from the breakdown of phospholipids for dietary absorption to the neuro- or cytotoxic action of venoms (Verheij et al., 1981a). These extracellular enzymes have some common characteristics: small size (~12-18 kDa) with about 120 amino acids in a single polypeptide chain; alkaline pH optimum; an absolute requirement for calcium (\( K_d \) values for calcium in the millimolar
These proteins have seven disulphide bridges that maintain a rigid three-dimensional structure. Due to the number of disulphide bonds, PLAs are highly resistant to denaturation (Dennis 1983; Verheij et al., 1981a). The extracellular enzymes are expressed at levels which allow the isolation of milligram quantities of protein from mammalian pancreas, reptile and insect venom. Extracellular PLAs have also been found in elevated levels in synovial fluid associated with inflammatory diseases such as arthritis (Kramer et al., 1989). The high molecular weight PLAs have a range of masses (the best characterised being approximately 85 kDa) and are located intracellularly. These proteins lack disulphide bridges since the cytosol is a reducing environment which would not favour disulphide bond formation (Clark et al., 1990). As with the extracellular enzymes, calcium is required for catalytic activity ($K_d$ values in the micromolar range) but they share no homology with the secretory proteins (Clark et al., 1991). The in vivo level of expression of these enzymes is low, which has hindered their isolation and characterisation. This group of enzymes is believed to play a role in the release of arachidonic acid, the twenty-carbon precursor of prostaglandins, leukotrienes and other biologically active eicosanoids. Eicosanoids are involved in signalling mechanism and inflammatory reactions (Moncada and Higgs, 1988; Samuelsson et al., 1987; Seilhamer et al., 1989).

Recently, other PLAs have been identified which do not depend on calcium for activity. Only a handful of these calcium independent PLAs (iPLAs) have been purified and none, as yet, have been sequenced. Thus, very little is known about the structure or mechanism of iPLAs or their relationship to the other known PLAs. iPLAs have been shown to exist in almost every mammalian cell tissue examined and some are localised to the lysosome (Ackermann and Dennis, 1995).

PLAs represent a paradigm of how a water soluble enzyme "recognises" and interacts with organised lipid aggregates. Phospholipids have no significant solubility in the aqueous phase, and so PLA$_2$ must catalyse the hydrolysis of the substrate at the lipid-water interface. Another feature of PLA$_2$ and lipases is the observed phenomenon of "interfacial activation". Unlike other esterases (such as horse liver esterase), PLA$_2$ displays very low activity when the substrate is present as monomers (Fig. 1.2). Once the substrate concentration has surpassed a critical concentration (known as the critical micelle concentration or cmc), organised
phospholipid aggregates form such as micelles or vesicles. PLA<sub>2</sub> shows a significant increase in activity (up to 10,000 fold increase depending on the substrate) at the organised lipid-water interface compared to monomer substrates. Interfacial activation is not seen with other esterases which show a normal Michaelis-Menten activity dependence on the substrate concentration (Verger and de Haas, 1976).

There is also interest in how the different extracellular enzymes display a clear preference for a particular aggregated substrate state. For example, the pancreatic enzymes prefer the substrate organised in mixed micelles whereas the venom enzymes favour bilayers. Clearly, these enzymes have evolved to recognise the \textit{in vivo} aggregated states of their particular biological targets, but considering the similarity in the primary amino acid sequence and tertiary structure the mechanism by which this is achieved is still unknown.
Finally, there has also been interest in understanding the role of PLA\textsubscript{2} in inflammation, immune events and intracellular signal transduction. This has arisen from studies showing that arachidonic acid (a twenty carbon fatty acid) is frequently found at the sn-2 position of phospholipids located in biological membranes. Thus the release of arachidonic acid by the action of PLA\textsubscript{2} has been shown to initiate an inflammatory response (Kramer \textit{et al.}, 1989).

Certain biologically active compounds such as the eicosanoids are synthesised primarily from arachidonic acid and include cyclooxygenase products such as thromboxanes and prostaglandins as well as lipooxygenase products such as leukotrienes (Irvine 1982; Samuelsson \textit{et al.}, 1987). These compounds have been associated with several inflammatory disorders such as arthritis (Samuelsson \textit{et al.}, 1987). Thus this may represent an important link between PLA\textsubscript{2} activity and the cascade of events that lead to the formation of inflammatory disorders.

1.2 Primary structure classification of extracellular PLA\textsubscript{2}s

The primary sequences of more than 60 extracellular PLA\textsubscript{2}s are known and have been found to be highly homologous (Dufton and Hider, 1983). There are a few exceptions, such as the bee venom enzyme which has little homology with the other enzymes and presumably evolved independently.

With a few exceptions, the extracellular PLA\textsubscript{2}s contain seven disulphide bridges (five of which are absolutely conserved) which maintain the conformation required for activity. If the disulphide bonds are reduced, there is a loss of enzyme activity, which is only restored after oxidation in the presence of low molecular weight thiol reagents to reform the disulphide bonds (van Scharrenburg \textit{et al.}, 1980).

Extracellular PLA\textsubscript{2}s can be further classified into three Groups (I, II and III), depending on several defined features, including their primary amino acid sequences and some structural differences, as suggested by Ackermann and Dennis (1995). Fig. 1.3 shows a sequence alignment of the Group I and Group II PLA\textsubscript{2}s.
Group I PLA$_2$s comprise the enzymes from pancreatic juice and the venoms of Elapid (cobra) and Hydrophididae (sea snakes). The Group I PLA$_2$s possess characteristic half cystines at residues 11 and 77 that are missing from the Group II enzymes.

The Group II PLA$_2$s include enzymes from the venoms of Crotalidae (rattlesnakes and pit vipers) and Viperidae (old world vipers). They lack the half cystines at amino acids 11 and 77, but have cysteine at 50 and at the C-terminus. In addition the Group II enzymes have extensions of about 6-8 residues beyond the corresponding C-terminus of the Group I enzymes. The Group II enzymes also have a large deletion between amino acids 57 and 66 (Renetseder et al., 1985). A major difference between the pancreatic and the venom enzymes is that the former are expressed as pro-enzymes which are activated via proteolysis (by trypsin) to remove a N-terminal heptapeptide.

The Group III PLA$_2$s include the evolutionary divergent venom enzymes from the European honeybee *Apis mellifera* (Kuchler et al., 1989), the Gila monster *Heloderma*
suspectum (Gomez et al., 1989), and the Mexican bearded lizard Heloderma horridum horridum Wiegman (Sosa et al., 1986).

PLA\(_2\)s isolated from different sources also have differences in the aggregated states of the purified enzyme. The pancreatic PLA\(_2\)s are isolated as monomers and do not aggregate except in the presence of strong anionic detergents (Arthur and Choy, 1989). Enzymes from Crotalidae are dimers with very little tendency to dissociate into monomers in solution (Renetseder et al., 1985). Although some enzymes have been isolated as dimers, work carried out by Jain et al has clearly shown that the secretory PLA\(_2\)s from venoms and pancreas were fully catalytically active as monomers (Jain et al., 1991a).

The tertiary structures of a number of Group I and Group II PLA\(_2\)s have been solved by X-ray crystallography. The structures indicate that regardless of the group, the tertiary structures are remarkably similar. Crystallographic studies of bPLA\(_2\) (Group I enzyme) and C. atrox (a Group II rattlesnake enzyme) have shown them to be structurally very similar (Wery et al., 1991; Renetseder et al., 1985). The αC-backbone of the two structures was shown to be very similar with a "homologous core" region being virtually superimposable (Fig. 1.4).

The regions which showed the greatest structural differences were shown to be related to the changes in the primary structure. The loss of Cys-11—Cys-77 bond and the replacement with one at Cys-50—Cys-133 resulted in the C. atrox enzyme assuming a more open orientation in the β-wings (residues 74-84) compared to the pancreatic enzyme. Residues 57-66 form a loop in the Group I enzyme. The absence of this loop in the venom enzyme has been suggested to be responsible for its dimerisation (Renetseder et al., 1985).
1.3 The structure of the pancreatic \( \text{PLA}_2 \)s.

The pancreatic enzymes (average molecular weight of 14 kDa) are approximately 22 x 30 x 42 Å in size, with 50% of the residues present in \( \alpha \)-helix and 10% in an antiparallel two stranded \( \beta \)-sheet. The core of the molecule consists of two antiparallel \( \alpha \)-helices (C and E in Fig. 1.5). The N-terminal region is also in an \( \alpha \)-helical structure (helix A) with the first amino acid residue buried in the interior of the protein. Helices B and D are short \( \alpha \)-helices of approximately one turn only. From the bovine X-ray crystal structure, helix D runs from
residues 59 to 66 (Dijkstra et al., 1981a), but this helix is not present in the porcine pancreatic structure, instead residues 59 to 66 have a random coil conformation and 67 to 71 form 1.5 turns of a 3_{10} helix (Dijkstra et al., 1983a; Thunnissen et al., 1993). Residues 74-78 and 81-85 form two antiparallel β-strands (often referred to as the β-wing) at the surface of the molecule, and the seven disulphide bonds maintain the compact structure of the molecule providing increased stability. The role of the disulphide bonds in the structure, conformational stability, and catalytic function of bPLA₂ have been investigated by site directed mutagenesis (Zhu et al., 1995). Each of the seven disulphide bonds was deleted in turn by changing both Cys residues to Ala, after which the protein was overexpressed in E. coli and refolded in vitro. Six of the disulphides did not appear to be important in the folding of the molecule into its native structure, as these mutants refolded to give similar quantities of enzyme as recombinant wild type protein. The Cys-84—Cys-96 mutant was unable to be refolded, suggesting that this disulphide bond may be important in the folding of the enzyme. The Cys-11—Cys-77 deletion produced the most unstable enzyme, suggesting its role in conformational stability. The effects on catalysis were limited for all of the mutants except Cys-29—Cys-45; the deletion of this disulphide bond caused a decrease in the catalytic activity, and this was attributed to a decrease in the ability to bind calcium (by a factor of 10), since the Cys-29—Cys-45 is involved in stabilising the calcium binding loop (Zhu et al., 1995).

The extracellular PLA₂S contain several structural features which possess defined functional roles within the enzyme, Fig. 1.5 shows the individual regions which will be discussed.
Fig. 1.5: Schematic diagram showing the secondary structure elements of bPLA₂ as determined by X-ray crystallography. The five α-helices (A,B,C,D and E) are shown, as is the β-wing. Key structural elements such as the seven disulphide bonds, the calcium binding loop, the Group I loop and the active site are shown.

1.3.1 The active site.

The active site of PLA₂ is located in a cavity at the molecule's surface. The residues involved in catalysis, His-48 and Asp-99, are located at the bottom of this cavity (the catalytic mechanism will be outlined in full in Section 1.5). His-48 has been shown to be involved in catalysis, as its modification with p-bromo-phenacyl bromide destroys enzymatic activity.
Chapter 1: Introduction.

(Volwerk et al., 1974). The wall of the active site is covered by hydrophobic residues forming a channel. For porcine and bovine PLA2s, the channel-forming residues are Phe-5, Ile-9, Phe-22, Ala-102, Ala-103, Phe-106 and the disulfide bond between Cys-29 and Cys-45 (Dijkstra et al., 1983a). Diffusion of the substrate through the hydrophobic channel is facilitated by a water-tight seal between conserved residues surrounding the channel mouth and the interface (Scott et al., 1990a). The hydroxyl group of Tyr-69 (which forms the mobile left flap of the hydrophobic channel) serves to fix and orientate the phosphate group of the phospholipid monomer in the active site (Kuipers et al., 1989; Scott et al., 1990a).

1.3.2 The amino-terminal helix (residues 1-12).

The first 12 amino acid residues of PLA2 form an α-helix (helix A) which performs several structural functions. The amino-terminal nitrogen of Group I and II PLA2s lies at the centre of an extensive network of hydrogen bonds that interconnect the amino-terminal helix with the active site of the enzyme (Fig. 1.6). A free N-terminal α-NH₃⁺ group is absolutely required for full catalytic activity of PLA2. Blocking the formation of this hydrogen bond network significantly affects activity and the ability of the enzyme to bind to aggregated substrates, for example when bPLA2 is transaminated. The transamination reaction, which is known to occur between glyoxylic acid and the α-amino group of proteins (Dixon and Fields, 1972), gives rise to a protein with an α-keto acid instead of an α-amino acid at the N-terminal part. Thus the N-terminal Ala of bPLA2 was converted into a pyruvoyl group by the selective modification of the α-NH₃⁺ to oxygen (Dijkstra et al., 1984). Therefore the N-terminal amino acid becomes chemically modified and is unable to form the active site hydrogen bond network.
Fig. 1.6: Schematic representation of the hydrogen bonding network involving the N-terminus and the active site of (A) bovine pancreatic PLA$_2$ and (B) porcine pancreatic PLA$_2$. The hydrogen bonds are shown (dashed lines), as is the N-terminal amino acid Ala-1 (bold) (modified from Dijkstra et al., 1983a). The bovine and porcine hydrogen bonding systems are similar, but they differ in the finer details. In the porcine enzyme, the N-terminus is somewhat more open to the solvent than in the bovine enzyme.
Chapter 1: Introduction.

The effect on the enzyme is a reduction in catalysis on micelles by two orders of magnitude, but no significant affect on the activity on monodispersed substrate (Dijkstra et al., 1984). The kinetic and structural characteristics of the transaminated bPLA₂ were shown to be similar to that of the zymogen. For example, from the X-ray crystal structure residues 1-3 and 63-72 of the transaminated enzyme were found to be highly mobile and disordered as seen in the pro-enzyme; and also the chemically modified protein, like the zymogen, could not bind to micelles of the neutral substrate analogue n-octadecylphosphocholine (Dijkstra et al., 1984; Pietserson et al., 1974). The zymogen has a heptapeptide located at the N-terminus of the protein, thus preventing the formation of the active site hydrogen bond network and inactivating the enzyme until the polypeptide is removed by the action of trypsin (Abita et al., 1972). As well as contributing several residues to the hydrophobic channel and to the active site, Helix A also forms part of the interfacial recognition site (IRS) (see Section 1.3.7).

1.3.3 The calcium binding loop (residues 25-42).

The pancreatic enzymes bind calcium relatively weakly, the dissociation constant (Kₐ) of calcium is 2.8 mM for porcine PLA₂ and 2.0 mM for bovine PLA₂ at pH 8.0 (Li et al., 1994; Tsai et al., 1985). The calcium ion has several functions: assisting binding of the substrate to the active site via an ionic interaction with the phosphate group (Yu et al., 1993); participation in the chemical step by the polarisation of the carbonyl group of the sn-2 ester linkage of the substrate and stabilisation of the transition state oxyanion (Scott et al., 1990a); and involvement in binding of the enzyme to the lipid-water interface via electrostatic interactions (Scott et al., 1994b; van den Bergh et al., 1989a). Thus the presence of the calcium ion is essential for the function of the enzyme.

The amino acid residues 25 to 42 form the primary calcium binding loop in PLA₂. The backbone carbonyl oxygens of residues 28, 30 and 32 co-ordinate the primary calcium ion as well as the carboxylate oxygens of Asp-49 and two water molecules, forming a tight bipyramidal co-ordination cage as shown in Fig. 1.7 (Dijkstra et al., 1983a; Bekkers et al., 1991). Upon binding a phospholipid molecule, the two water molecules are displaced and their positions occupied by the ligand (Scott et al., 1990a). Several other highly conserved
residues in the 25-42 loop serve to stabilise the conformation of the calcium binding site (Dijkstra et al., 1981a).

![Diagram of calcium binding pocket](image)

**Fig. 1.7:** Schematic representation of the calcium binding pocket of bovine pancreatic PLA$_2$ (modified from Dijkstra et al., 1981a).

A secondary calcium binding site has been identified in both porcine and equine PLA$_2$s, but it is absent from the bovine enzyme (van Sharrenburg et al., 1984; van den Bergh et al., 1989; Donné-Op den Kelder et al., 1983). It has been shown that the secondary binding site has a relatively low affinity for calcium, for example for pPLA$_2$, $K_{d}$~20 mM at pH 7.5 (Slotboom et al., 1978). The secondary calcium site is located near the N-terminus, and for pPLA$_2$ residues Glu-71 and to a lesser extent Asp-66, are involved in binding the divalent ion (van den Bergh et al., 1989). The secondary calcium ion was found to be kinetically important for pPLA$_2$ at pH 7 and above. At higher calcium concentrations the affinity of the enzyme for the lipid-water interface at basic pH values was improved (van Dam-Mieras et al., 1975). Whether or not the second calcium binding site has any role *in vivo* is still unknown, as very little is known about the physiological conditions in the intestine. But the high pH values at which the second site becomes functional and the high calcium concentrations required to restore the interaction with micelles do not suggest any major physiological significance (van den Bergh et al., 1989).
1.3.4 Helices C (residues 37-54) and E (residues 90-109).

These two major helices form the core of the enzyme and contribute the catalytic residues. The residues involved in catalysis are highly conserved between all PLA₂S, and consist of a His at position 48 and Asp at position 99 (the catalytic mechanism will be outlined in full in Section 1.5). The helices are also highly disulfide bridged, five (Group I) or six (Group II) of the disulfide bridges found in the extracellular PLA₂S incorporate half-cystines derived from these substructures.

1.3.5 The Group I loop (58-71).

Group I PLA₂S feature a distinctive loop of surface exposed residues arising from the distal tip of the first antiparallel helix. This loop is absent in the Group II enzymes, moderately developed among the Group I elapids, and prominent among the Group I pancreatic enzymes. The greatest difference in the secondary structure that occurs between the bovine and porcine enzymes is within the 58-71 loop. Although the primary sequence contains only two amino acid differences (porcine Phe-63 → bovine Val-63 and porcine Glu-71 → bovine Asn-71), the X-ray crystal structures clearly show that the secondary structure is different in the 58-71 surface loop (Dijkstra et al., 1983a; Dijkstra et al., 1983b). The loop structure in pPLA₂ consists of a random coil conformation (59-66), followed by a short 3₁₀-helix (67-70), whereas in bPLA₂ residues 59-66 adopt an α-helix conformation, while residues 67-70 are in a surface loop (Dijkstra et al., 1983a). It has been shown by Thunnissen et al., (1993) that mutation of Phe-63 in pPLA₂ to a Val (as in bPLA₂) appears to change the conformation of the surface loop. The loop has a greater structural similarity to the bovine enzyme than the porcine protein as determined by X-ray crystallography. The affinity of binding to micelles and the catalytic rate had been reduced, but the mutant was still more similar to the porcine enzyme than the bovine protein (Thunnissen et al., 1993).

One of the largest differences in the secondary structure between the elapid venom and the pancreatic enzymes is the deletion of five amino acid residues (the group I loop) in the former enzyme. The snake venom enzymes have a higher affinity for the lipid-water interface.
than the pancreatic enzymes and they also hydrolyse monomolecular substrates at a greater rate. In an attempt to improve the catalytic properties of pPLA₂, a series of site directed mutagenesis experiments were performed where residues 62-66 were deleted, Ser-60 was changed to Gly and Asn-67 to Tyr to increase the similarity to the venom enzyme (C. atrox) (Kuipers et al., 1989). The deletion of residues 62-66 from pPLA₂ improves activity on micellar zwitterionic phospholipids up to 16-fold (Kuipers et al., 1989). The increased affinity for zwitterionic phospholipids was probably due to the formation of a more favourable surface for interfacial absorption. The mutants’ activity on negatively charged substrates decreased and activity on monomeric phospholipids increased relative to the native enzyme. Thus the specificity and kinetics of the mutant pPLA₂ were changed to those similar to the venom enzyme. Associated with the kinetic changes the structure of the enzyme also showed similarities to the venom enzyme at the IRS (see Section 1.3.7) as determined by crystallography. It was speculated by Renetseder et al., (1985) that the Group I loop may prevent the pancreatic enzymes from forming dimers by steric hindrance. Beiboer et al., (1995) demonstrated that the loop was involved in dimerisation as deletion of residues 62-66 from pPLA₂ induced protein aggregation.

1.3.6 The β-wing (74-84).

The Group I and II enzymes contain a single loop of antiparallel β-sheet called the β-wing (see Fig. 1.5). Sequence conservation within this region is poorly maintained between the Group I and II enzymes, but the underlying conformation is highly maintained across species. The most significant difference within this region is the presence (Group I) or absence (Group II) of a disulphide bond; the absence of the disulphide bond in the Group II proteins allows for greater flexibility of the β-wing. The β-wing in the Group II enzymes is somewhat stabilised by an ion pair interaction, for example in C. atrox PLA₂ Lys-11 forms a long noncovalent link to the side chains of Glu-77 and Glu-78 through several water molecules (Renetseder et al., 1985).
1.3.7 The interfacial recognition site.

Bovine and porcine PLA$_2$s show significant differences in their affinities towards lipid-water interfaces. pPLA$_2$ can degrade more densely packed monolayers of phospholipids than the bovine enzyme (Dutilh et al., 1975; van Scharrenburg et al., 1981). To explain the interaction of the enzyme with its aggregated substrate, Pietersen et al., (1974) proposed that the protein possessed an interfacial recognition site (IRS). The IRS or i-face, is the surface of the protein molecule which interacts with the aggregated substrate (e.g. micelles). The IRS is separate from the active site since inactivation of the enzyme (using para-bromophenacylbromide which reacts with His-48) at the active site still allows the molecule to bind to the interface (Volwerk et al., 1974). Kinetic and amino acid modification experiments combined with the three-dimensional structure of bPLA$_2$ helped to identify which residues were part of the IRS (Dijkstra et al., 1983a). These residues were: Leu-2, Trp-3, Asn-6, Glu-17, Leu-19, Leu-20, Asn-23, Asn-24, Leu-31, Lys-56, Val-65, Asn-67, Tyr-69, Thr-70, Asn-72, Lys-116, Asn-117, Asp-119, Lys-120 and Lys-121 (Fig. 1.8). Thus the N-terminal helix, the primary calcium binding loop, the Group I loop and the C-terminus all contribute residues to the IRS.

The presence of positively charged residues has been correlated to the preference of the pancreatic enzymes for negatively charged aggregated substrates (van Scharrenburg et al., 1981; Dijkstra et al., 1983a). It has been shown that these residues are not absolutely conserved in all the PLA$_2$ sequenced so far. Five substitutions have occurred at the IRS between the bovine and porcine enzyme and they are at positions 6 (Asn in bovine$\rightarrow$Arg in porcine), 17 (Glu$\rightarrow$His), 20 (Leu$\rightarrow$Met), 72 (Asn$\rightarrow$Ser) and 120 (Lys$\rightarrow$Thr). The porcine enzyme also has two other residues which are part of the IRS. They are Lys-121, due to an amino acid insertion and Leu-64, due to the conformation of the surface loop 58-71. Thus these changes at the IRS may explain the differences in affinity for the lipid-water interface between the two pancreatic enzymes.

Both electrostatic and hydrophobic interactions have been shown to be important in absorption of PLA$_2$ to the lipid-water interface.
Fig. 1.8: The amino acid residues which have been identified as part of the IRS of bovine pancreatic PLA₂. The structures shown are (A) the molecule as seen face-on and (B) from the side. The hydrophobic and charged residues surround the active site, and form the surface which interacts directly with the lipid-water interface.
Chapter 1: Introduction.

Generally, the affinity of the enzyme for lipid-water interfaces can be improved by the introduction of a positive charge at the IRS of the protein, or a negative charge to the surfactant interface. For example, the substitution of Asn-6 for Arg in bPLA₂ resulted in a two to three fold increase in activity on mixed micelles of egg-yolk lecithin with deoxycholate and on neutral micelles of 1,2-dioctanoyl-sn-glycero-3-phosphocholine (van Scharrenburg et al., 1983). The substitution of a positive charge for a negative one (Arg-6->Glu-6) at the same position in pPLA₂ had the opposite effect, whilst the activity on neutral micelles of 1,2-dioctanoyl-sn-glycero-3-phosphocholine was reduced by a factor of four (de Haas et al., 1987). Surfactants added to the interface have also been shown to increase (anionic compounds) or decrease (cationic compounds) the catalytic rates of the enzyme (Volwerk et al., 1986).

The ability to penetrate the more densely packed mono- or bilayer structures can also be affected by introducing hydrophobic groups at the IRS. Generally the pancreatic enzymes do not penetrate phospholipid bilayers to the same extent as the venom enzymes, but replacing Leu-20 with a Trp residue in bPLA₂ enhanced the ability of the protein to penetrate densely packed neutral monolayers and bilayers (Lee et al., 1996). A Trp residue is found at position 20 in many snake venom PLA₂s (e.g. *Naja naja naja* PLA₂).

1.4 Human synovial fluid PLA₂.

Group II PLA₂ (which is also referred to as non-pancreatic, synovial or secretory PLA₂) is found in several tissues as a secretory and a cell surface associated enzyme, for example in placenta, platelets, neutrophils and peritoneum. This group of enzymes are also able to bind heparin and heparin sulphate which could provide a mechanism for scavenging circulating PLA₂ and for protein binding to the extracellular matrix or cell membranes (Murakami et al., 1992). Some cell types such as chondrocytes, vascular smooth muscle cells and renal mesangial cells actively secrete Group II sPLA₂ after treatment with inflammatory cytokines such as interleukin (IL)-1 or tumour necrosis factor (TNF) (Kudo et al., 1993). The enzyme has a wide range of functions *in vivo* as summarised in Table 1.1.
Chapter 1: Introduction.

<table>
<thead>
<tr>
<th>Physiological function</th>
<th>Pathological role</th>
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<tr>
<td>Fat digestion</td>
<td>Rheumatoid arthritis</td>
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<td>Lung surfactant metabolism</td>
<td>Psoriasis</td>
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<td>Cell membrane homeostasis</td>
<td>Inflammation</td>
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<td>Insulin release</td>
<td>Premature labour</td>
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<td>Sperm maturation/penetration</td>
<td>Septic shock/endotoxin reactions</td>
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<td>Lipoprotein metabolism</td>
<td>Renal disease</td>
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<td>Haemostasis and blood clotting</td>
<td>Inflammatory bowel disease</td>
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<td>Hypertension</td>
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Table 1.1: Physiological and pathological processes in which PLA₂ or a product of PLA₂ activity has been implicated. Most of the studies characterised specifically sPLA₂ (Glaser et al., 1993).

The interest in sPLA₂ lies in the fact that the fatty acid arachidonate is frequently located at the sn-2 position of many membrane phospholipids. Hydrolysis of these phospholipids by sPLA₂ generates a lysophospholipid and arachidonic acid, both of these molecules are involved in the initiation of cascades that control key biochemical pathways in vivo. For example, the lysophospholipid hydrolysis product can be metabolised to produce platelet-activating factor (PAF). PAF is an important regulator of cellular functions, especially those of the endothelium. The arachidonic acid released can also be metabolised by cyclooxygenases and lipoxygenases to produce various pro-inflammatory eicosanoids compounds such as prostaglandins, leukotrienes and thromoxanes. These molecules are known mediators of inflammation, hypersensitivity and tissue injury (Moncada et al., 1988; Samuelsson et al., 1987). sPLA₂ links with various disease states has been implicated through the isolation of high levels of the enzyme at the site of the disorder (Table 1.1). For example, multiple forms of sPLA₂ have been purified from the synovial fluid drained from the joints of patients suffering from rheumatoid arthritis (RA) (Seilhamer et al., 1988; 1989). It is thought that the pathophysiological reactions are mediated by the eicosanoids and platelet-activating factor. Further evidence for the involvement of sPLA₂ in inflammatory diseases has come from experimental observations. It can be shown that both membrane-associated and secreted (soluble) forms of sPLA₂ are present in and produced by cells participating in the inflammatory reaction. The same enzymes when sequestered in peritoneal or joint cavities may accumulate to very high levels in diseases such as RA. Animal models have been used to
show that injection of purified sPLA$_2$ into knee joints, at concentrations found in vivo, cause acute inflammation and disease-like symptoms (Kramer et al., 1989; Seilhamer et al., 1989; Mizushima et al., 1989). Group II PLA$_2$ has also been shown to enhance eicosanoid generation from activated neutrophils and mast cells (Mayer and Marshall, 1993), and binds specifically to cell membranes of platelets, neutrophils, chondrocytes and macrophage-like cells. Thus it has been speculated that chronic inflammation may arise from a positive feedback loop. For example, sPLA$_2$ increases the level of cytokine production from T-lymphocytes, the cytokines in turn up-regulate PLA$_2$ secretion from a second cell type, thus sustaining chronic inflammation as seen with RA (Mukherjee et al., 1994). The link between the presence of sPLA$_2$ and a variety of disorders and the fact that the enzyme is the rate limiting step in the production of the mediators of inflammation, makes sPLA$_2$ a good target for a specific inhibitor. There is a problem with the development of such an inhibitor; it must be specific to sPLA$_2$ and have a low affinity for the pancreatic enzyme and have few side effects. A wide range of competitive and transition-state inhibitors are available for in vitro use, however no specific PLA$_2$ inhibitory drugs are presently in use in clinical medicine. One promising compound is an indol based compound which binds to the active site of sPLA$_2$ (Schevitz et al., 1995). The inhibitor is related to the well-known anti-inflammatory drug indomethacin (Schevitz et al., 1995), which is a potent cyclooxygenase inhibitor and has been reported to weakly inhibit rabbit and human sPLA$_2$ (Lobo et al., 1994). It has been shown that the inhibitor molecule had a 1,500 fold preference for sPLA$_2$ over porcine pancreatic PLA$_2$ (Schevitz et al., 1995). The prolonged exposure to such an inhibitor would have to be carefully monitored as the long term physiological effects of sPLA$_2$ inhibition are unknown.

The sPLA$_2$ enzyme has been classified as a Group II enzyme since it lacks both Cys-11 and Cys-77, but has the extra disulphide bridge between Cys-50 and the C-terminus extension Cys. The largest difference between sPLA$_2$ and the bovine pancreatic enzyme occurs in the 57-68 loop; a region of high structural diversity and where the latter protein has an additional six residues which form a helix. The X-ray crystal structure has shown that the $\alpha$C-backbone of sPLA$_2$ has a greater similarity to the structure of the venom enzyme (C. atrox) than to the pancreatic enzymes (Wery et al., 1991). Like the venom enzymes, sPLA$_2$ is secreted in its active form i.e. without an N-terminal prosequence which has to be removed for activity.
sPLA$_2$ is a very positively charged molecule with 23 arginines and lysines. There are only eight glutamic and aspartic acid residues, with two of the latter, Asp-49 and Asp-99, required for function. Thus sPLA$_2$ is a basic enzyme (pI value greater than 10.5) and also has positively charged amino acids near its N-terminus (Arg-7, Lys-11 and Lys-15). Such positively charged residues have been proposed to be important in the interaction of sPLA$_2$ with specific biological targets and the enzymes’ preference for negatively charged substrates such as phospholipids in *E. coli* membranes (Bayburt *et al.*, 1993).

1.5 The catalytic mechanism of PLA$_2$.

The catalytic mechanism has been studied using the crystal structures of *Naja naja atra* and the evolutionary distant bee-venom PLA$_2$ complexed with a phosphonate transition-state analogue (Scott *et al.*, 1990a; Scott *et al.*, 1990b; White *et al.*, 1990). The functionally important and invariant residues His-48, Try-52, Tyr-73 and Asp-99 from these extracellular PLA$_2$s are superimposable on those of both bovine pancreatic and *C. atrox* PLA$_2$ (Renetseder *et al.*, 1985). Hence the hydrogen-bonding network that connects these residues is virtually identical and thus the catalytic mechanism will be the same for all PLA$_2$s.

Fig. 1.10 shows the proposed binding and catalytic mechanism. The first step (A) involves the formation of an attacking nucleophile from a water molecule (water 218) (Wery *et al.*, 1991; Scott *et al.*, 1990a) which is hydrogen bonded to N81 of His-48. His-48 abstracts a proton from water 218 which simultaneously attacks the carbonyl function of the scissile ester. The hydroxyl group of water 218 then forms part of the tetrahedral intermediate created during catalysis. The oxanyion of the tetrahedral intermediate which is formed becomes a ligand of the calcium ion which, with the backbone amide NH from Gly-30 stabilises the intermediate (B). The final stage (C) involves the protonation of the *sn*-2 oxygen and the collapse of the tetrahedral intermediate into its products. Three water molecules move into the active site to replace the products. One will engage the N81 of His-48 and the remaining two will co-ordinate the calcium ion.
Fig. 1.10: The proposed catalytic mechanism of Group I/II PLA$_2$ (modified from Scott et al., 1990a). The substrate shown is 1-pentanoyl-2-monanoyl-sn-glycero-3-phosphoethanolamine.
1.6 The organisation of aggregated phospholipids.

Phospholipids are amphiphilic molecules, i.e. they possess both hydrophobic and hydrophilic characteristics. In aqueous solution phospholipids may form micelles or bilayered structures such as vesicles (Fig. 1.11). These molecular arrangements eliminate unfavourable contacts between water and the hydrophobic tails, but permit solvation of the head group. An assembly of a few amphiphiles cannot shield its acyl chains from contact with water. Therefore, dilute solutions of surfactants do not form micelles until their concentration passes the critical micelle concentration (cmc). Above the cmc, most of the surfactant aggregates to form micelles. The value of the cmc depends on several factors such as the number of carbon atoms in the acyl chains (as the chain length increases the cmc decreases), temperature, salt concentration and pH.

Cone shaped amphiphiles such as fatty-acids and lysophospholipids form spheroidal micelles, whereas the steric requirements of packing short-chain synthetic phospholipid molecules together (e.g. 1,2-dioctanoyl-sn-glycero-3-phosphocholine) yields large dislike micelles such as ellipsoids. Aqueous dispersions of naturally occurring phospholipids (cylindrical-shaped) form bilayer-enclosed vesicles (liposomes). Addition of a micelle forming detergent above a certain concentration to vesicles of long-chain phospholipids leads to the destruction of vesicles and the formation of mixed micelles.

Short chain phospholipids have been especially useful in studying PLA$_2$ as they have enabled the kinetic behaviour from monomers through to micelles to be investigated (as these synthetic phospholipids have high cmcs) (Roberts, 1991). By using phospholipids with different head-groups or by the addition of a detergent, a variety of charged interfaces can be produced. For example, phosphatidy/cholines possess both a negative (phosphate) and positive charge and therefore the micelles will be of overall neutral charge. But if the choline group is substituted for a glycerol group the overall charge is negative, as is the charge at the interface. Negatively and positively charged detergents (e.g. cholate and cetyltrimethylammonium bromide respectively) can also be added to change the charge at the lipid-water interface in the mixed micelles (Volwerk et al., 1986).
Chapter 1: Introduction.

There are important differences between micelles and vesicles, for example small vesicles contain a few to several thousand phospholipids, whereas micelles are much smaller containing between 10-200 amphiphiles. Also, the concentration of monomer phospholipids in the aqueous phase in equilibrium with vesicles is very low (less than 100 pM for naturally occurring phospholipids), compared to micelles where the concentrations range from μM to mM (Jain and Berg, 1989). The rate of vesicle fusion, intervesicle exchange or transbilayer "flip-flop" of phospholipids is negligibly small (half-times greater than 10 hours to several days). Lateral diffusion of phospholipids within bilayers is a rapid event compared to the flip-flop of molecules. In order for the enzymatic activity of PLA$_2$ to be limited by lateral diffusion the turn-over number would have to exceed 200,000 s$^{-1}$ (Jain and Berg, 1989). With mixed micelles transfer of long-chain phospholipids between micelles occurs on a time-scale of seconds, even though half-times for intermicelle exchange of detergent and short-chain amphiphiles is of the order of $10^{-4}$ s (Gelb et al., 1995). Since long-chain phospholipids do not desorb from micelles into aqueous solution, a different mechanism for the transfer of phospholipids from one micelle to another is required. Intermicelle exchange requires the collision and fusion of micelles to give transiently formed particles in which phospholipid transfer can occur, followed by fission to re-form stable mixed micelles (Gelb et al., 1995).

The ability of PLA$_2$ to recognise the correct organised state of the phospholipid is crucial to the function of the enzyme since the biological target of the pancreatic enzymes will be in the form of mixed micelles and that of the venom enzymes in the form of bilayers. Care must therefore be taken to choose the correct physical state of the substrate in enzyme assays and kinetic studies, for example the densely packed phospholipid monomers in bilayers are not hydrolysed efficiently by the pancreatic enzymes (unlike the venom PLA$_2$s) as they are unable to penetrate the interface (Lee et al., 1996).
Chapter 1: Introduction.

MICELLES

Hydrophobic acyl chain

Hydrophilic head-group

BILAYERED VESICLE

Liposome

Mixed micelles

(i)

(ii)

Fig. 1.11: The formation of ordered aggregated surfactant structures such as micelles and bilayers. The two surfactants are examples of molecules that form (i) bilayers, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and (ii) micelles, α-hexadecylphosphocholine.

1.7 Enzyme activation at the lipid-water interface.

Extracellular PLA₂s bind both monomolecular and aggregated phospholipid structures. Their activity on monomeric substrates is low, but is increased by several orders of
Chapter 1: Introduction.

on the formation of organised phospholipid structures such as micelles and bilayers. Lipids form these larger structures once the concentration has passed a certain point (the cmc). Thus it appears that PLA₂ is able to recognise organised lipid interfaces, bind to them, and have a much higher activity. This increase in activity is termed "interfacial activation" (Section 1.1; Fig. 1.2). The venom enzymes do not exhibit interfacial activation and have a high activity even on monomeric substrates. This has been attributed to the formation of protein-lipid aggregates below the cmc. Thus any model describing the mechanism of interfacial activation must explain the enzymes' low activity on monomer substrates and the enhancement at the lipid-water interface. Three theories have been proposed to explain the activation of PLA₂ at water-lipid interfaces:

i) The enzyme theory assumes that upon interacting with the interface, there is a conformational change in the enzyme that is eventually translated into increased activity.

ii) The substrate theory suggests changes in the phospholipid molecule in its transformation from a monomer to an aggregated form makes it more susceptible to hydrolysis by the enzyme.

iii) The concentration effect suggests that at the lipid-water interface the enzyme in effect is exposed to a higher substrate concentration that brings the enzyme closer to saturation.

The models with the most experimental evidence are (i) and (ii). The enzyme theory is often referred to as the interfacial recognition site (IRS) model and has been supported by experiments carried out by de Haas and co-workers, Jain et al., and Tsai et al. The substrate theory is mainly based on conclusions developed from the X-ray crystal structures of PLA₂s complexed with inhibitors by Sigler and co-workers.

1.7.1 The interfacial recognition site (IRS) model.

This model was proposed by de Haas and co-workers for pancreatic PLA₂ and is based on the enzyme theory. They proposed that PLA₂ contains a region on the surface of the
Chapter 1: Introduction.

molecule (the interfacial recognition site or IRS) which binds reversibly to the lipid-water interface. On binding, a conformational change occurs within the enzyme which results in the optimal alignment or exposure of the active site residues to the substrates leading to enhanced hydrolysis. This activation is shown in Fig. 1.12.

According to the above scheme, the enzyme free in solution (E) undergoes a structural change on binding to the lipid-water interface and becomes activated (E*). Once at the interface the enzyme binds a substrate molecule (S) to form the enzyme-substrate complex (E*S). The substrate is hydrolysed and the product (P) is formed, after which the enzyme is free to hydrolyse further phospholipid molecules or (depending on the affinity for the lipid-water interface) leave the micelle/vesicle (see Section 1.8). Further evidence for the existence of various states of PLA₂ has been provided by Jain and Maliwal (1993), who showed that the E, E* and E*L (L being a non-hydrolysable ligand) forms of the enzyme have distinct spectroscopic signatures. The spectroscopic differences between the protein species were due to the localised environment around Trp-3, which is located at the IRS and thus interacts directly with the lipid-water interface. The E* form of the enzyme was studied by binding the protein to micelles of a non-hydrolysable neutral diluent (1-hexadecylpropanediol-3-
Chapter 1: Introduction.

A neutral diluent is defined as an amphiphilic molecule that forms an aggregate to which PLA\textsubscript{2} can bind via its IRS. However the affinity of a molecule of neutral diluent for the active site of the enzyme is low so that the active site remains ligand-free (filled with water). Thus absorption to the interface and substrate binding are two separate events.

The residues which are thought to form the IRS are listed in Section 1.3.7. There is evidence that shows that both electrostatic and hydrophobic interactions play a crucial role in interfacial recognition of the lipid-water interface (see Section 1.3.7).

Triacylglyceride lipases are another family of enzymes that show interfacial activation at a lipid-water interface. These lipases hydrolyse ester bonds in neutral lipids, the catalytic residues being Ser, His and Asp or Glu, like the catalytic triad found in the serine proteases. Unlike PLA\textsubscript{2}, in which no significant conformational difference between the native and inhibited enzyme is observed in the X-ray crystal structures (Scott et al., 1990a), all the lipases studied so far have their catalytic residues buried beneath one or more surface loops. Using X-ray crystallography, a lipase isolated from \textit{Rhizomucor miehei} (RmL) was shown to contain a short amphipathic helix which acted as a "lid". Upon inhibition with a covalently bound inhibitor, this helix moves away from the active site across the surface of the protein and as a result a large hydrophobic surface becomes exposed (Derekwenda et al., 1993).

More recently, nmr has been used to determine the structure of porcine PLA\textsubscript{2} free in solution and in a ternary complex (Peters et al., 1992; van den Berg et al., 1995a; 1995b). The ternary complex consisted of the enzyme bound to a micelle (dodecylphosphocholine) and the active site occupied by a phosphonate transition state analogue. The enzyme was isotopically labelled with \textsuperscript{13}C and \textsuperscript{15}N to solve the problem of signal overlap in the \textsuperscript{1H} nmr spectrum due to the high \alpha-helical content of the protein. It was found that the enzyme free in solution had regions which were disordered, such as the 58-71 surface loop and the N-terminus as shown in Fig. 1.13 (van den Berg et al., 1995a; 1995b). Since the N-terminal \alpha-amino group was located between 8-11 Å from Tyr-52 and Asp-99, this excluded the possibility of the formation of the hydrogen bonding network involving the active site residues. Thus the catalytic residues, His-48 and Asp-99, appeared to be disordered (Peters et al., 1992; Dekker et al., 1991; van den Berg et al., 1995a; 1995b; 1995c). The structure of the enzyme free in solution
Chapter 1: Introduction.

appeared to be similar to the zymogen X-ray crystal structure which also has several poorly
defined regions of the secondary structure (e.g. the 58-71 surface loop). The tertiary structure
of the enzyme was found to be very similar to the published X-ray crystal structures. So the
structures of PLA₂ as determined by X-ray crystallography may represent the enzyme at the
lipid-water interface. Therefore the absence of the hydrogen bonding network between the α-
amino group and the active site, and the conformational freedom of the catalytic residues, may
explain the low enzymatic activity on monomeric substrates (van den Berg et al., 1995b).

Activation of the enzyme when bound to a micelle may be due to a substrate molecule binding
at the active site which induces the α-helical conformation of the N-terminus and the
formation of the hydrogen bonding network within the active site. Consequently the enzyme
adopts a more rigid conformation (as seen in the X-ray crystal structures), and enhanced
activity is observed as the catalytic residues become ordered (van den Berg et al., 1995b).

Fig. 1.13: (A) The 20 conformers of porcine PLA₂ free in solution as determined by nmr, the 58-71 surface loop
is shown. (B) The average nmr solution structure (van den Berg et al., 1995b). The N and C terminus are also
shown.
Jain et al have also shown that the N-terminal helix is involved in binding at the interface. In free solution the N-terminal segment is disordered and is thought to exist as an ensemble of conformations. They proposed that a diverse population of structures is required to facilitate binding to a variety of lipid-water interfaces, since the precise organisation of the interface differs from one substrate to another. Binding of the enzyme causes the N-terminus to form a rigid helix which brings about the desolvation of the interface and the active site and facilitates the diffusion of lipid monomers from the vesicles to the active site (Jain and Maliwal 1993; Maliwal et al., 1994; Liu et al., 1995). Further support for the mechanism of the substrate diffusing into the active site was shown by Soltys et al., (1993), who synthesised cross-linkable short-chain phosphatidylcholines with thiols at the chain terminus. When reduced and above the cmc, the thiol lipids were excellent substrates for *Naja naja naja* PLA$_2$. Upon polymerisation (by oxidation using H$_2$O$_2$), the micelles were found to be very poor substrates for PLA$_2$ and this was attributed to the inability of the substrate to move out of the plane of the interface.

1.7.2 The substrate theory.

Scott *et al* have suggested that PLA$_2$ does not undergo any major structural changes upon binding to the interface (i.e. activation as outlined by the substrate theory rather than the enzyme theory of interfacial activation). This hypothesis was derived from the X-ray crystal structure of the venom enzyme (*Naja naja atra*) in complex with a phosphonate transition-state analogue (Scott *et al.*, 1990a). A comparison of the uninhibited form of the *N. n. atra* enzyme with that of the enzyme-transition state analogue complex showed no change in the $\alpha$C-backbone structure of the enzyme. It was postulated that the monomeric substrate free in solution exists in a variety of conformers, but only a very small proportion of the phospholipids have the correct conformation which can bind to the active site and be hydrolysed by the enzyme. But on passing the cmc, the substrate is forced into a conformation, by the micelle or vesicle, which facilitates diffusion into the active site of PLA$_2$ through the hydrophobic channel of the enzyme. Thus PLA$_2$ has low activity on monomer substrates and
is only activated once at the lipid-water interface due to the high concentration of optimally disposed substrate available.

PLA$_2$ has a hydrophobic channel into which monomeric substrate diffuses from the micelle or vesicle towards the active site residues (Fig. 1.14). Diffusion of the substrate molecule through the hydrophobic channel is facilitated by a water-tight seal between the residues around the active site and the interface. The left wall of the channel is formed by a potentially mobile hydrophobic "flap" that is only secured firmly once the substrate is productively bound. In the *Naja naja atra* venom enzyme the wall is the aromatic ring of Tyr-69 whose phenolic hydroxyl is bound to the inhibitor's sn-3 phosphate. Some members of the Group I/II family have a Lys-69 residue, in which case the four methylene groups of this side chain probably form the hydrophobic left wall of the channel and the ε-amino group binds the sn-3 phosphate. The conformation of the substrate in the complexes is similar to that seen in crystalline aggregates as well as that inferred by nmr experiments on phospholipids in micelles. Thus the enzyme may have evolved a mechanism in which it specifically selects for the conformation imposed upon the phospholipid by the aggregate.

Fig. 1.14: Schematic representation of PLA$_2$ at the lipid-water interface. Diffusion of the substrate is facilitated by the water-tight seal between the residues surrounding the active site and the interface (modified from Scott *et al.*, 1990a). The stabilisation of the transition state of the reaction by the essential calcium ion is shown.
1.7.3 The concentration effect.

The concentration effect was proposed by Brockman et al., (1973) to explain the increased activity of PLA₂ at the lipid-water interface. The existence at the interface of an ordered array of lipid molecules in a high local concentration could create a situation similar to the concentration of lipid molecules in a pure bulk lipid phase. This should allow more frequent productive collisions with a soluble enzyme molecule than could occur for monomeric lipid molecules tumbling free in solution. However, it seems difficult to explain reported rate increases of $10^3$-$10^4$ by only a concentration rise, and it is also difficult to understand why esterases behave so differently from lipases which do not exhibit an increase in activity as the substrate concentration increases past the cmc (see Fig. 1.2). A second objection to the concentration effect arises from the Michaelis-Menten model of catalysis at the micelle interface (Fig. 1.12). According to Michaelis and Menten, in a monomeric solution of substrate the maximal velocity is reached when all enzyme in solution is in the ES form. This would also be the upper limit if the enzyme was present at the interface. If the complexes ES (bulk) and E*S (interface) were identical, it is hard to imagine accelerating factors of 1000 and more due to the local concentration of substrate.

1.8 Kinetic characterisation of PLA₂₆.

Interfacial catalysis represents a key characteristic of extracellular PLA₂₆, but does produce problems in the kinetic analysis of the enzyme at the lipid-water interface. A large number of secreted PLA₂₆ bind much more tightly to anionic vesicles than to neutral vesicles (van Oort et al., 1985; Volwerk et al., 1985), thus the charge at the interface (produced by the phospholipids or added surfactants) has a significant effect on protein absorption. The difference in affinity for the lipid-water interface produces two extreme modes of interfacial catalysis, "scooting" and "hopping" (Fig. 1.15).
Chapter 1: Introduction.

Fig. 1.15: The overall kinetic mechanism of PLA₂ at a lipid-water interface (modified from Jain and Berg, 1989). The abbreviations are: enzyme free in solution (E); the enzyme activated by the lipid-water interface (E*); the substrate (S); the enzyme substrate complex (E*S); and the products of the reaction (P) (see Section 1.7.1 for details). The two extreme modes of hydrolysis are also shown. In the highly processive scooting mode of catalysis, only the phospholipid present at the interface of the occupied vesicle can be hydrolysed (dashed line). If the enzyme is in the hopping mode of catalysis, then the enzyme has the opportunity to hydrolyse all the substrate present, as the enzyme is able to leave the lipid-water interface and reabsorb onto the same or another vesicle.

The above scheme is a simple adaptation of the Michaelis-Menten representation of enzyme kinetics at interfaces. Different interfaces exhibit a difference in the kinetics of hydrolysis because they vary in their ability to shift the equilibrium from E→E*, thus depending on the affinity for the interface the enzyme will either be in the highly "processive" scooting mode, or the less active hopping mode. During the scooting mode of catalysis, the enzyme remains at the surface of the vesicle hydrolysing all the phospholipid molecules in the outer envelope. If the enzyme is in hopping mode of catalysis, the enzyme once it has turned over a substrate molecule, has the ability to remain or leave the lipid-water interface (as its
Chapter 1: Introduction.

affinity for the interface is low). Thus if the enzyme is in the hopping mode of catalysis it has
the opportunity to eventually hydrolyse all the phospholipids in the outer layer of all the
vesicles present (Fig. 1.15).

Kinetic characterisation of PLA₂ has been complicated by these two modes of
catalysis as the key assumption in steady state kinetics is that all the enzymes within the
sample experience the same experimental conditions, for example, the same substrate
concentration. Even when bound to the aggregated substrate the enzyme only "sees" a small
proportion of the total substrate in the reaction mixture. It can be seen that during hopping
mode catalysis, there will be several populations of enzyme molecules in different
environments. A proportion of the enzyme will be absorbed at the lipid-water interface and
another population will be free in solution. This distribution will be determined by the E→E*
equilibrium, which is determined by the phospholipid composition of the lipid-water interface.

Since the enzyme can move from one interface to another in the hopping mode, the substrate
concentration available for hydrolysis will also vary from enzyme to enzyme depending on
the concentration of phospholipid at the present vesicle interface. Therefore there may be
localised regions which contain more enzyme, and for these the build-up of product and the
onset of product inhibition would occur earlier in time compared to regions where the enzyme
concentration is lower. In order to fully account for all the characteristics of hopping mode
catalysis a considerable increase in the complexity of the kinetic theory would be necessary.

The above problems of describing interfacial kinetics has been simplified by ensuring
that the enzyme is only in the scooting mode of catalysis. This work has been carried out by
Jain et al, using vesicles of the synthetic phospholipid 1,2-dimyristoyl-sn-glycero-3-
phosphomethanol (DMPM) with pPLA₂. This has allowed the measurement of the rate
parameters of interfacial catalysis by fitting the reaction progress curves to Michaelis-Menten
equations modified for interfacial catalysis (Jain et al., 1986b; 1986c; Jain et al., 1991a;
1991b; 1991c; 1991d; 1991e; Berg et al., 1991). There are several advantages of using
DMPM over other phospholipids. Firstly, PLA₂ has a high affinity for vesicles of DMPM
compared to zwitterionic vesicles, and this is reflected in the $K_d$ for the two types of interfaces
which is 0.1 pM for DMPM compared to more than 1 mM for vesicles of
dimyristoylglycerophosphocholine (DMPC) (Jain and Berg, 1989a). It has been shown that
once the substrate present in the outer bilayer of the DMPM vesicle has all been turned over by pPLA$_2$, any vesicles of DMPM subsequently added to the reaction mixture are not hydrolysed, suggesting that the enzyme cannot leave the interface and that vesicle fusion does not occur (Jain et al., 1986a; 1986b; 1986c). Thus with vesicles of DMPM, PLA$_2$ remains at the interface exclusively in the scooting mode of catalysis which greatly simplifies the kinetic analysis. The size of the DMPM vesicles can be varied, for example small vesicles (with ~4,000 phospholipid molecules in the outer surface) can be produced by sonication, but more importantly large vesicles can be produced by extrusion through membranes housed in stirred flow cells (Berg et al., 1991). These large vesicles contain between ~10,000-100,000 phospholipid molecules in the outer surface (depending on the pore size of the filter used), which allows the enzyme's activity to be followed for several minutes. DMPM vesicles were found to be very stable (>8 hours) and do not fuse, or allow the exchange of substrate or products from the interface to other vesicles, even after all the phospholipid in the outer bilayer had been hydrolysed (Jain et al., 1986a; 1986b; 1986c). Vesicle fusion can be initiated by the addition of divalent cations, abrupt changes in pH, or by the addition of a fusogen such as polymyxin B (Jain et al., 1991d). Finally one other experimental constraint must be adhered to when investigating interfacial binding parameters. The concentration of vesicles must be at least five times greater than that of the enzyme ensuring that on average only one protein molecule is present per vesicle. These requirements need not be met if only the initial reaction velocity is sought.

Therefore using DMPM vesicles has allowed the investigation and measurement of the rates of key steps in interfacial catalysis by ensuring that the enzyme remains at the lipid-water interface in the highly processive scooting mode. Under these experimental conditions, all the enzyme molecules should "experience" the same environment and so this allows the complex nature of interfacial kinetics to be simplified and to be interpreted using standard Michaelis-Menten theory.
1.9 Substrate specificity of \( \text{PLA}_2 \).

\( \text{PLA}_2 \) can hydrolyse a wide range of phospholipids (with a variety of head-groups and acyl chain lengths), but at different turnover rates. Thus it would appear that \( \text{PLA}_2 \) shows a preference for a particular type of phospholipid aggregate, for example choline derived substrates are hydrolysed at greater rates than ethanolamine substrates. But Ghomashchi et al., (1991) have shown that this is incorrect as the observed preference when using pure phospholipid vesicles/micelles is determined by the ability of the particular interface to shift the \( E \rightarrow E^* \) equilibrium. So enzymatic velocities that are measured in separate experiments using different vesicles, each composed of a single molecular species, will mainly reflect the relative affinities of the enzyme to the different vesicles. Therefore, the substrate preference can only be investigated when in the scooting mode of hydrolysis. DMPM vesicles have thus proved to be ideal in determining substrate specificity. This has been achieved by embedding small amounts of competing substrates (e.g. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and 1,2-dipalmitoyl-sn-glycero-3-phosphate) into a vesicle of DMPM (Ghomashchi et al., 1991). The competing phospholipids are labelled with different radioactive isotopes such as \(^{3}\text{H} \) and \(^{14}\text{C} \) so that the amount of each species hydrolysed can be measured. The DMPM vesicle ensures that the enzyme will be in the scooting mode of hydrolysis at the interface where it will now have a choice of substrates. These studies showed that the 14 kDa secreted \( \text{PLA}_2 \) do not significantly discriminate against phospholipids of differing head-groups or acyl chains. This is completely different to the results obtained if one compares the relative velocities of the enzyme acting on vesicles composed of different pure phospholipids where the enzyme's rate reflects the ability of the different lipid-water interfaces to shift the \( E \rightarrow E^* \) equilibrium. Therefore with micelles of pure phospholipids a comparison of the "IRS substrate specificity" rather than "active site substrate specificity" is being observed.

1.10 Site-directed mutagenesis of \( \text{PLA}_2 \).

An important method of investigating the structure/function relationships of enzymes has been by the use of site-directed mutagenesis (SDM). This is achieved by manipulating the
Chapter 1: Introduction.

DNA sequence of the gene, so that upon translation one or more specific residues are changed within the primary amino acid sequence. Characterisation of the structure and function of the mutant enzyme may provide an insight into the role of the original residue.

Numerous PLA$_2$ mutants have been made using SDM to investigate the role of individual, or a series of, residues. In the case of PLA$_2$ this may provide information on the function of the residue(s) in i) interfacial recognition, ii) the catalytic mechanism, iii) protein stability, iv) calcium binding or v) phospholipid binding at the active site. Due to the relatively small size of the protein, a single mutation usually affects more than one of the above functions. For example, to investigate the structural role of conserved aromatic residues in enzyme stability, Phe-22 and Phe-106 of bPLA$_2$ were individually substituted for isoleucine, alanine and tyrosine (Dupureur et al., 1992a). Phe-22 and Phe-106 are highly conserved residues and arranged so that they form part of an invariant hydrophobic wall that shields the active site of the enzyme from bulk solvent (Dijkstra et al., 1981a; 1981b). These residues have also been suggested to interact with the $sn$-2 acyl chain of the bound phospholipid substrate (White et al., 1990). The results of the mutations showed that i) all the single mutants had similar conformational stability to that of the wild type enzyme, as determined by the ability of guanidine-hydrochloride to denature the proteins. Thus Phe-22 and Phe-106 do not play any major structural roles. ii) Isoleucine substitutions at 22 or 106 resulted in only minor perturbations in activity, suggesting that the aromaticity was not important in the function of these two residues. iii) Alanine substitutions at either of these positions resulted in a 10-200 fold decrease in the catalytic activities, suggesting that the hydrophobic bulk of both residues was important for catalysis, possibly by interacting with the $sn$-2 acyl chains. iv) The Tyr-22 mutation had no effect, whereas Tyr-106 decreased the enzyme's activity. This may be because of the orientation of the side chains: Phe-22 points outwards facing solvent, whereas Phe-106 points into the active site. It was concluded that the perturbation in the function of F22A, F106A and F106Y was due to a change in $k_{cat}$; binding of the enzyme to the interface and binding of ligands to the enzyme at the interface were less perturbed (Dupureur et al., 1992a).

The importance of hydrophobic interactions in protein stability and activity were also apparent from SDM of Tyr-52 and Tyr-73 residues. The conserved amino acids Tyr-52 and
Tyr-73 were investigated to clarify their functions in the active site of pPLA2 (Thunnissen et al., 1992) and bPLA2 (Sekharudu et al., 1992). These two tyrosine residues form hydrogen bonds to the carboxylate group of the catalytic Asp-99 and indirectly to Ala-1 through a water molecule (see Fig. 1.6). Sekharudu et al. substituted Tyr-52 and Tyr-73 in bPLA2 for Phe producing both the single and double mutants. The presence of phenylalanine residues would eliminate the hydrogen bonds that exist between the phenolic hydroxyl groups of Tyr-52 and -73. It was thought that these hydrogen bonds were essential for the interfacial recognition and the stability of the overall catalytic network. However, it was found that there was only a marginal reduction in the activity of the mutants when compared to the wild type enzyme, and a slight decrease in the protein stability. From the X-ray crystal structure of the double mutant it was evident that four hydrogen bonds had been lost; three involving the tyrosines and one involving Pro-68 (see Fig. 1.6a). The hydrogen bonds of the catalytic triad, His-48, Asp-99 and the catalytic water were retained. It was found that the substituted phenylalanine residues shifted to fill the voids left by the phenolic OH groups, thus increasing the hydrophobic interactions and compensating for the missing hydrogen bonds. This offered an explanation to why the double mutant had activity which was similar to the wild type enzyme and showed that hydrophobic interactions were important in maintaining the structure and stability of the active site and also the interfacial binding site (Sekharudu et al., 1992). The idea that large hydrophobic groups were important at positions 52 and 73 in PLA2 was also supported by the work of Dupureur et al., (1992b) on bPLA2. Substitution of Tyr-73 by alanine or serine residues reduced the catalytic activity by two to three orders of magnitude. Thus the void created by the entire phenyl group could not be filled by the smaller aliphatic residues. The Y73K mutant also had very low activity compared to the wild type enzyme. Although lysine could fill the void left by the phenyl group (to a certain degree), the positively charged ε-ammonium group of K73 destabilised the enzyme by the charge repulsion with the N-terminal ammonium group of Ala-1 (Dupureur et al., 1992b). The mutations were also introduced into a pPLA2 mutant in which residues 62-66 had been deleted (Δ62-66) because of its better crystallisation properties (Kuipers et al., 1989). The Y52F and Y73F mutants of Δ62-66 pPLA2 also possessed similar binding and catalytic properties to the Δ62-66 enzyme, but stability against denaturation by guanidine hydrochloride was decreased (Thunnissen et al.,
Unlike the bPLA2 tyrosine mutants, no conformational changes occurred in the Y52F and Y73F mutants of Δ62-66 pPLA2 as determined by X-ray crystallography. Instead an empty cavity was present at the site of the hydroxyl group of the former tyrosine. Therefore, either the formation of the cavity within the protein or the loss of one hydrogen bond with Asp-99 may explain the observed destabilisation of the mutant enzymes. Cavities in a protein molecule are energetically unfavourable because their surrounding residues have less van der Waals interactions than if these residues were located in a more densely packed environment.

The active site of the enzyme has also been investigated using SDM. To study the role of the Asp-99 and His-48 pair in bPLA2 catalysis, Asp-99 was mutated to asparagine (D99N) (Kumar et al., 1994). The mutant enzyme was still catalytically active, but with a ~370 fold reduction in activity on micelles of diC18:PC when compared to the native protein. X-ray crystallography showed that the carbonyl group of the Asn-99 side chain was hydrogen bonded to His-48 in the same way as that of Asp-99 in the wild type enzyme, thus retaining the native tautomeric form of His-48 and the function of the enzyme. Unexpectedly, it was found that a water molecule which performs a structural role in the hydrogen bonding network of the active site was missing. The loss of the water molecule allowed the NH2 group of Asn-99 to point away from His-48 and so preserve the hydrogen bonding pattern of the catalytic triad and the tautomeric form of His-48 as in the wild type enzyme. To adapt to the loss of the water molecule, the NH2 group of Asn-99 formed a direct hydrogen bond with the carbonyl of Ala-1 and so the enzyme was still able to bind to micellar interfaces (Kumar et al., 1994).

The primary calcium binding loop is highly conserved among many secretory PLA2s, the side chain carboxylate of Asp-49, along with the carbonyl oxygens of Tyr-28, Gly-30 and Gly-32, and two water molecules provide the necessary ligands for the calcium ion which is essential for enzymatic activity. The role of Asp-49 in bPLA2 has been studied by substituting this residue with an asparagine, a glutamic acid, a glutamine, lysine and alanine (Li et al., 1994). It was found that the mutations introduced did not affect the stability of the protein when compared to the wild type enzyme. Direct calcium binding, which was followed by UV difference spectroscopy, showed that the residues alanine, asparagine, lysine and glutamine at position 49 were unable to specifically bind Ca2+. However, D49E had a 12-fold weaker binding affinity for Ca2+ compared to the native enzyme (Li et al., 1994). The inability to
bind calcium was also reflected in the low specific activities of the mutants. This
demonstrated the functional importance of Asp-49 in the catalytic mechanism of PLA\textsubscript{2}. It was
presumed that the function of Asp-49 was to bind, and to correctly orientate the calcium ion.
Fluorescence binding studies showed that the mutants were all able to bind to zwitterionic
micelles and anionic vesicles (i.e. the E→E\textsuperscript{*} step). The D49A mutant was discovered to be
unable to bind active site directed ligands at the interface, thus showing that Asp-49 has a role
in ligand binding to the active site (i.e. the E\textsuperscript{*}→E\textsuperscript{*L} step) (Li \textit{et al.}, 1994). Similar results for
calcium binding and catalysis were obtained from the Asp-49 mutants of pPLA\textsubscript{2}, where
mutants were constructed which contained glutamic acid or lysine at position 49 (van den
Bergh \textit{et al.}, 1988).

The crucial role of the N-terminal helix in interfacial recognition and catalysis has
been studied by methods such as SDM and chemical modification (e.g. transamination). The
N-terminal helix contributes residues to the interfacial recognition site, the hydrophobic
channel and the hydrogen-bonding network of the active site. Residues at positions 2, 3, 4, 5,
6 and 9 of bPLA\textsubscript{2} were substituted to study their structure/function relationships (Liu \textit{et al.},
1995). Residues 2, 5, and 9 are thought to contribute to phospholipid binding through
interactions with the hydrophobic acyl chain of the substrate (White \textit{et al.}, 1990; Scott \textit{et al.},
1990a), the side chains of Phe-5 and Ile-9 form part of the wall of the hydrophobic channel.
Nonconservative mutations (e.g. alanine) of Phe-5 and Ile-9 led to significant reductions in the
stability of the mutant enzymes. Kinetic studies also showed that the mutations had reduced
the rates of hydrolysis on micelle and vesicle substrates. For example, the \textit{k\textsubscript{cat}} of authentic
bPLA\textsubscript{2} with micelles of diC\textsubscript{8}PC was determined to be 680 s\textsuperscript{-1}, whereas the \textit{k\textsubscript{cat}} of F5A and
I9A were found to be 13 and 20 s\textsuperscript{-1} respectively (Liu \textit{et al.}, 1995). The effects of the
mutations at positions 5 and 9 suggested that these residues are important for both structure
and catalysis. Leu-2 mutants appeared to show a distinct ability to discriminate between short
chain (C\textsubscript{8} in length) and long chain (C\textsubscript{14}) phospholipids. For example, the L2W mutant has
similar activity to native bPLA\textsubscript{2} with diC\textsubscript{8}PC or diC\textsubscript{8}PM, but there was a significant
reduction with the longer acyl chain of diC\textsubscript{14}PM. This may reflect the interaction of Leu-2
with the acyl chain of the substrate, the presence of larger side chains prevents this interaction
with C\textsubscript{14} phospholipids due to steric clashes. Gln-4 is a highly conserved residue in PLA\textsubscript{2}. 

\textit{Chapter 1: Introduction.}
and is involved in the hydrogen bonding network with the α-ammonium of Ala-1. Q4A only had small effects on catalysis suggesting that the hydrogen bond between Gln-4 and the N-terminus is not important for catalysis. Residues 3 and 6 have been generally accepted as part of the interfacial binding site of PLA₂ that includes the N-terminal helix. The effects on binding and catalysis of mutating Asn-6 and Trp-3 were modest, for example a three fold decrease in binding of the N6D mutant was measured with micelles of diC₅PC (Liu et al., 1995). This supported the theory that the interfacial binding site involves a large number of residues (Dijkstra et al., 1981b). Thus a change in one of them would have only an incremental change in the underlying kinetic parameters, rather than an all or nothing effect.

The Group I surface loop (residues 58-71) is known to contribute several amino acids to the IRS and is also absent from the elapid snake venom, and non-pancreatic enzymes. To try to improve the catalytic properties of pPLA₂ to that of the venom enzymes, the residues 62-66 were deleted (Kuipers et al., 1989). In redesigning the primary sequence, other substitutions were made to maintain maximum sequence homology with the elapid enzymes in the region of the loop (Kuipers et al., 1989). These simultaneous substitutions were D59S, S60G and N67Y, and the enzyme was referred to as Δ62-66 PLA₂. The structure of the mutant enzyme was determined by X-ray crystallography and it was found that the region from residues 58-70 of Δ62-66 PLA₂ was intermediate between those in porcine and C. atrox PLA₂. The catalytic activity of the mutant was greater than authentic pPLA₂ on a wide range of micellar substrates, the most significant was with diC₅PC. Δ62-66 PLA₂ had a 16-fold greater turnover rate with this synthetic phospholipid than pPLA₂, but this was still an order of magnitude lower when compared to Naja melanoleuca venom PLA₂ (Kuipers et al., 1989). The effect of the deletion and substitutions may have affected part of the putative binding site for phospholipid aggregates: Asn-67 was replaced by a tyrosine residue, Val-65 was deleted, and the X-ray analysis showed that Tyr-69 in the mutant enzyme had a position and orientation that differed from those of the wild-type enzyme. All of these changes could cause a different orientation of the active site of the mutant with respect to the lipid aggregate, allowing for a more efficient interaction with the individual phospholipid molecules, and thus increased catalysis.
Chapter 1: Introduction.

1.11 Aims of the project.

Over the last 25-30 years a vast amount of information has been gathered on a wide range of PLA\textsubscript{2}s isolated from a variety of sources. A key challenge is to identify the structural characteristics of these enzymes which are responsible for the molecular recognition/interactions with aggregated phospholipid substrates. PLA\textsubscript{2} offers an opportunity to investigate how a protein recognises and interacts with lipid-water interfaces, and in particular how a group of enzymes with such a high degree of structural similarity are able to distinguish between the different aggregated forms of phospholipids that exists (e.g bilayers, micelles and vesicles). The pancreatic PLA\textsubscript{2}s have evolved to recognise their substrates in the form of negatively charged mixed-micelles, since this is the aggregated state of the substrate which is formed during digestion. The snake venom enzymes are also very specific and prefer bilayers of zwitterionic lipids (e.g. phosphatidylcholine).

The initial work was to develop a protocol for the refolding and purification of human sPLA\textsubscript{2} which had been overexpressed in \textit{E. coli}. Once this had been achieved, mutants of sPLA\textsubscript{2} were to be produced in which the surface regions with the greatest diversity between sPLA\textsubscript{2} (which prefers bilayers) and bPLA\textsubscript{2} (which prefers mixed-micelles) were to be exchanged. The effect of these mutations on binding to a variety of charged lipid-water interfaces would be investigated using lipids in various aggregate states. The enzymology of the recombinant enzyme would be compared to sPLA\textsubscript{2} overexpressed in a mammalian expression system (murine erythroleukaemia cells or MEL cells). The solution structure of the wild type enzyme was also to be determined using nmr and isotopically labelled protein ($^{15}$N and $^{13}$C). With nmr, the interactions of sPLA\textsubscript{2} with inhibitors and detergent micelles would also be investigated in an attempt to identify characteristics which may be of use when considering the design of possible drug molecules targeted against sPLA\textsubscript{2}.

Work was also carried out on bPLA\textsubscript{2} to investigate the difference in kinetic and structural properties between bovine and porcine pancreatic PLA\textsubscript{2}s. The two enzymes only differ by 19 amino acid residues, but have different affinities for the lipid-water interface. pPLA\textsubscript{2} binds with a greater affinity to zwitterionic interfaces than the bovine enzyme and shows a much greater activity on mixed micelles.
The initial work was to improve the methods of expression, refolding and purification of recombinant bPLA2 from *E. coli* which had been achieved by Dr. H. Kogelberg. At the start of this work, purified protein yields of less than 2 mg/L of culture were obtainable, which were insufficient for economically viable isotopic labelling. Ultimately, with the ability to produce large quantities of isotopically labelled protein, the full solution structure determination of the enzyme could be completed (approximately 65% of the 1H nmr spectrum of bPLA2 has been assigned by Kilby *et al.*).

To investigate the difference in affinity between the bovine and porcine enzyme in binding to lipid-water interfaces, mutations in the Group I surface loop (residues 58-71) were made. This work was complementary to that carried out by Thunnissen *et al.*, (1993), who made the F63V mutation within the highly conserved 58-71 surface loop in pPLA2 in order to determine if this single point mutation was responsible for the large conformational difference observed between the bovine and porcine enzymes within this region. It was shown by X-ray crystallography that Phe-63 was responsible for the structural difference between the porcine and bovine enzymes, as the conformation of the 58-71 surface loop of F63V was similar to bPLA2. But the F63V mutant still had the binding and kinetic characteristics of the porcine enzyme. Therefore, although Phe-63 was thought to determine the conformation of the 58-71 surface loop, this residue was not responsible for the greater affinity of pPLA2 for lipid-water interfaces.

Since the bovine enzyme has a significantly lower affinity for lipid-water interfaces than the porcine protein, any bovine mutants which had porcine-like properties would be easily identified. Therefore site-directed mutagenesis was used to investigate what contributions residues Val-63 and Asn-71 in the highly conserved 58-71 surface loop made to interfacial recognition. This would also allow us to investigate whether these residues were involved in the binding and kinetic differences seen between bovine and porcine pancreatic PLA2. Three mutants were produced to see if the structure of the surface loop and binding affinity for the lipid-water interface could be changed from bPLA2 to that of pPLA2. Therefore the two residues in the 58-71 surface loop of bPLA2 were changed to those found in the porcine enzyme, i.e. V63F, N71E and the double mutant, V63FN71E. The effects of these mutations were determined by using enzyme assays and fluorescence spectroscopy with a
variety of phospholipids and surfactants. The effects of these mutations on the overall structure of the enzyme (especially the 58-71 surface loop) will eventually be investigated by nmr using isotopically labelled protein.
CHAPTER 2
MATERIALS AND METHODS

2.1 Chemicals and reagents.

Chemicals and reagents were purchased from the following suppliers:

Aldrich- 0.1 N sodium hydroxide.

Amicon- Centricons, Microcons and membranes for stirred-flow cell concentrators (10 K and 3 K cut-off).

Boehringer Mannheim- Factor Xa protease and Taq DNA polymerase

Calbiochem- Hexadecylphosphocholine and any remaining phospholipids not purchased from Sigma.

Cambio- DNA PCR-ladder (50-1000 bp).

Cascade Biochem Ltd- HEPC

Fisons- Microtitreplates, 5 L round bottom flask, Nalgene filter units and all plastic ware.

Gibco-BRL- Ultra-pure agarose, Lambda phage DNA, T4 DNA ligase and Dulbecco's modified Eagle media (containing 4.5 g/L glucose and without sodium pyruvate).

Hybaid- Qiagen tip-100 plasmid DNA purification kit.

ICN- Foetal Bovine serum.

Life Technologies Inc.- DH5α E. coli cells.

New England Biolabs- Deep Vent DNA polymerase, Taq DNA Ligase, T4 polynucleotide kinase and the vector Bluescript II SK .

Novagen- All remaining E. coli bacterial cell lines and pET vectors (Studier et al., 1990).

Oligonucleotides- Oligonucleotides were synthesised using an Applied Biosystems 394 oligo-synthesiser by Dr. K. Lilley.

Orme scientific equipment- Spectra/por 1 dialysis tubing (M₄ cut-off 8 kDa).
Chapter 2: Materials and Methods.

Pharmacia- Ultrapure dideoxy NTPs, deoxy NTPs, T7 DNA polymerase and Fast Flow S-Sepharose resin.

Promega- All restriction enzymes, T4 DNA polymerase and calf intestinal alkaline phosphatase.

Sigma- diC₃PC and diC₅PC, and all other remaining chemicals, solutions and enzymes.

Stratech Scientific LTD- Gene Clean II kit.

Water- Distilled water and Super Q water were purified using the appropriate Millipore water purifying equipment.
Chapter 2: Materials and Methods.

METHODS

2.2 Molecular biology.

General molecular biological procedures are described in full in Appendix A.

2.2.1 PCR mutagenesis.

A PCR strategy was used to produce mutants of bPLA2. The general method is shown schematically in Fig. 2.1. The DNA primers used to produce the bPLA2 mutants are shown in Table 2.1. The first stage involves two PCR reactions which separately amplify the 5' and 3' ends of the gene (using the appropriate forward and reverse primers, see Table 2.1). The amino acid point mutation was introduced by designing the internal primers to contain the changed codon sequence. The mutation was placed at the centre of the oligonucleotide.

Prior to use, the oligonucleotide primers were isopropanol precipitated (Appendix A3.7) from the ammonium solution and DNA concentrations were determined from the A260 measurements (see Appendix A3.9). In a 100 µL PCR reaction 7.5 fmoles (25 ng) of pbPLA2-pET12a (5.07 Kb) was used as the DNA template, 0.4 µM of each primer, 10 µL of 10x Deep Vent buffer and 200 µM of each dNTP (Appendix A2.1). One unit of Deep Vent DNA polymerase was added last to minimise the degradation of primers by the 3'-5' exonuclease activity of the enzyme. 40 µL of PCR oil was layered onto the reaction mix to prevent evaporation. A control PCR which did not contain any template DNA was carried out to check for DNA contamination. The PCR cycling conditions used were:- denaturation 1 minute (92°C); annealing 30 seconds (55°C); and primer extension for 30 seconds (72°C) for 25 cycles.
Fig 2.1: A schematic representation of the procedure for the introduction of point mutations into bovine pancreatic PLA₂.
<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Length (bases)</th>
<th>$T_m$ (°C)</th>
<th>DNA sequence (5'-3')</th>
<th>Mutation (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Universal primer 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27</td>
<td>58.1</td>
<td>AAA CTC CTA GGA ATA GTC CTG ACA ACC</td>
<td>-</td>
</tr>
<tr>
<td>Reverse Universal primer 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28</td>
<td>58.4</td>
<td>G CTT GGA TCC TTA GCA GTT TTT TTT GTC</td>
<td>Lys122→Lys122 TTT→CTT</td>
</tr>
<tr>
<td>Forward V63F-1</td>
<td>27</td>
<td>59.6</td>
<td>CTG GAC TCC TGC AAA TTT CTG GTT GAC</td>
<td>Val63→Phe63 GTT→TTT</td>
</tr>
<tr>
<td>Reverse V63F-2</td>
<td>27</td>
<td>59.6</td>
<td>GTC AAC CAG AAA TTT GCA GGA GTC CAG</td>
<td>Val63→Phe63 AAC→AAA</td>
</tr>
<tr>
<td>Forward N71E-1</td>
<td>27</td>
<td>61.2</td>
<td>AAC CCG TAC ACC GAG AAC TAC TCC TAC</td>
<td>Asn71→Glu71 AAC→GAG</td>
</tr>
<tr>
<td>Reverse N71E-2</td>
<td>27</td>
<td>61.2</td>
<td>GTA GGA GTA GTT TTT GGT GTA CGG GTT</td>
<td>Asn71→Glu71 GTT→CTT</td>
</tr>
</tbody>
</table>

**Table 2.1:** Oligonucleotides used in the production of mutants of bPLA<sub>2</sub> (the point mutations are shown in bold type).

**Notes:**
- <sup>a</sup>The $T_m$ values were determined by the program MacVector™.
- <sup>b</sup>The primer anneals 90 bp upstream of the gene within the ompT region of the plasmid.
- <sup>b</sup>See Chapter 5 Discussion Section 5.8, for a full explanation of Lys-122 sequence change.
Chapter 2: Materials and Methods.

After purifying the products by electrophoresis in a 1.5% agarose gel followed by electroelution (Appendix A3.2 and A3.4), a third PCR reaction was used to fuse the 5' and the 3’ gene fragments together (Fig. 2.1, Reaction 3). 0.2 nM of each of the products was used in this reaction along with 0.4 μM of each universal primer. The reaction conditions were as described previously, except the extension time was extended to 1 minute at 72°C. The full length mutagenic product (460 bp) was isolated by electrophoresis in a 1.5% agarose gel followed by electroelution (Appendix A3.2 and A3.4). 0.5 μg of the DNA was digested with Sal I and BamH I overnight (Appendix A2.2), and the 370 bp fragment isolated and ligated downstream of the ompT secretory signal of pET12a (4.5 Kb). The vector DNA had been previously digested with Sal I and BamH I sequentially and then treated with calf intestinal alkaline phosphatase (Appendix A2.3). 25 fmoles of vector and 75 fmoles of insert DNA were ligated overnight at 16°C in the presence of T4 DNA ligase (Appendix A2.5) and the ligation mix was then used to transform 100 μL of competent DH5α E. coli cells (Appendix A3.11). Transformed cells were selected on LB agar plates (Appendix A1.2) containing ampicillin (50 μg/mL) grown at 37°C overnight.

bPLA2 mutants V63F, N71E and V63FN71E were produced as outlined above, using the mutagenic oligonucleotide primers V63F-1, V63F-2, N71E-1 and N71E-2. bPLA2-pET12a plasmid template was used in the production of V63F and N71E, whereas V63F-pET12a was used for V63FN71E.

2.2.2 PCR screening for gene insertion into the pET12a vector.

Bacteria containing the vector/gene construct were screened using PCR. Single colonies were picked (using a sterile tip) from the transformation plate and transferred to a marked LB agar plate containing ampicillin (50 μg/mL) so that they could be identified later. The plate was then incubated overnight at 37°C. The same tip was used to transfer the remaining cells to a 20 μL PCR reaction containing 0.25 μM forward and reverse universal primers and 1 unit of Taq DNA polymerase. This PCR would therefore specifically amplify the bPLA2 gene. The PCR cycling conditions used were: denaturation 1 minute (92°C); annealing 30 seconds (55°C); and primer extension for 1 minute (72°C) for 25 cycles. 10 μL
of the product was run out on a 1.5% agarose gel. Cells carrying the vector-gene construct were identified by the presence of a 460 bp band; a PCR ladder was used to determine the molecular weight of the products. Bacterial colonies which had been identified as carrying the bPLA2 gene in pET12a were picked from the marked agar plate and used to inoculate 5 mL of LB media containing ampicillin (50 µg/mL). These small scale cultures were used to make glycerol stocks and stored at -20°C (Appendix A3.12).

2.2.3 Single base 35S dideoxynucleotide termination sequencing.

To increase the number of clones that could be screened, single base sequencing was carried out. This allowed the identification of clones which incorporated the mutagenic oligonucleotide sequence. C-tracking was used since a cytosine base was expected to be missing for the Val→Phe-63 mutation (AAC→AAA) and two to appear for the Asn→Glu-71 mutant (GTT→CTC) in the non-coding DNA strand when compared to the wild-type sequence.

5 mL of LB media (100 µg/mL ampicillin) was inoculated with cells carrying the mutagenic plasmid. Five clones were grown overnight at 37°C. The cells were harvested by centrifugation (5,000 x g for 10 minutes) and the plasmid DNA isolated using the mini-prep method (Appendix A3.8). From 5 mL of culture =1-2 µg of plasmid DNA was isolated, which was then denatured using the alkaline denaturation protocol (Appendix A4.1) to give single stranded plasmid DNA. After annealing the appropriate sequencing primer to the single stranded DNA, the target DNA was sequenced using the T7Sequencing kit, following the manufacturers protocol. Only the 35S dideoxyctydine termination reactions were carried out. The sequencing reactions for the two mutants were run next to wild-type DNA for a direct comparison of the sequence. Two clones for each mutant which had been identified as having the correct C-banding pattern were then fully sequenced.
2.2.4 ³⁵S dideoxynucleotide termination sequencing.

Plasmid DNA for sequencing was purified using Qiagen-tip-100 columns. Cells harbouring the plasmid were grown in 150 mL of LB containing 100 µg/mL ampicillin overnight at 37°C. The plasmid DNA was purified as recommended by the manufacturer, giving between 50-90 µg of DNA. Single stranded plasmid was produced by alkaline denaturation (Appendix A4.1), after which the appropriate sequencing primers were annealed as described in the T7Sequencing kit protocol. Both coding and non-coding DNA strands were fully sequenced using the T7Sequencing kit protocol (Appendix A4.2).

Once the correct mutant DNA sequences had been identified the plasmids were used to transform the *E. coli* protein expressing strain BL21 (DE3) pLysS. The overall cloning procedure is shown in Fig. 2.2.
Chapter 2: Materials and Methods.

pET 12a vector

Vector digested with *BamH I* and *Sal I* and phosphatase treated.

PCR product

PCR product digested with *BamH I* and *Sal I*

+ 

Ligation using T4 DNA Ligase

Transform into *E. coli DH5α* cells

Select for transformed cells with antibiotics

Screen for cells with vector/insert DNA using PCR

Grow large scale cultures for $^{35}$S DNA sequencing

Wild type | Mutant
---|---

$^{35}$S DNA sequencing gel using a single base reaction

Site of mutation

Clones containing the mutagenic oligonucleotide sequence are then fully sequenced.

Fig. 2.2: Overview of the cloning and sequencing procedures used to produce and identify bPLA$_2$ mutants.
Chapter 2: Materials and Methods.

2.3 Production of pro-sPLA₂ for expression in MEL cells.

2.3.1 Construction of the pro-sPLA₂ gene.

The vector used for the DNA manipulations was pBluescript II SK (2.96 Kb), which utilises a blue/white selection procedure for positive clones on AXI plates (LB agar, 100 μg/mL ampicillin, Xgal 0.08 mg/mL and 1 mM IPTG). The secretory signal was added to sPLA₂ so that it would be efficiently secreted from the MEL cells into the culture supernatant. Once the correct construct had been identified in Bluescript, it was then cloned into the MEL cell expression vector pEVS (13.7 Kb).

The sPLA₂ cDNA, which also contained the N-terminal factor X₄ cleavage recognition site (pro-sPLA₂ or psPLA₂) had been previously made by Dr. P. Mallinder in the vector pUE. 5 μg of pUE psPLA₂ was digested with EcoR I and Sal I (Appendix A2.2) and then loaded onto a 0.8% agarose gel to purify the 400 bp psPLA₂ gene by electroelution (Appendix A3.2 and A3.4). The pBluescript vector, which had previously been digested with EcoR I and Sal I, was treated with alkaline phosphatase (Appendix A2.3) before phenol extraction and isopropanol precipitation of the DNA (Appendix A3.6 and A3.7). The psPLA₂ fragment (75 fmole) was ligated into the EcoR I/Sal I cut vector (25 fmole) with an insert to vector molar ratio of 3:1. The ligation was carried out at 16°C overnight using T4 DNA ligase (Appendix A2.5). The ligation mixture was then used to transform competent DH5α E. coli cells (Appendix A3.11). The cells were plated out onto AXI plates and grown overnight at 37°C. Several white colonies were picked from the plate and grown for plasmid mini-preps. Double digestion of the plasmid DNA using EcoR I and Sal I, which produced a 400 bp band on an agarose gel, was carried out to identify positive clones. The cloning of the secretory signal sequence into the psPLA₂ Bluescript vector was achieved using PCR. The signal sequence was constructed by four overlapping oligonucleotides (Table 2.2 and Fig. 2.3). The 5' end of AB1 contains a EcoR I cloning site. The 3' end of AB3 was designed so that it could be cloned into and disrupt the EcoR I site at the 5' end of psPLA₂ Bluescript. The signal sequence also contains a unique Nco I site and a ribosome binding site, i.e. a Kozak sequence (Kozak, 1986).
<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Length (bases)</th>
<th>( T_m ) (^\text{(^{\circ}\text{C} )})</th>
<th>Sequence (5'(\Rightarrow)3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1</td>
<td>35</td>
<td>61.0</td>
<td>CTAGTGAAATTCCACATAGACACCTCCCTACTUTT</td>
</tr>
<tr>
<td>AB2</td>
<td>47</td>
<td>65.8</td>
<td>GATCATGATCGCAAGCAAGGAGGTCTTGATGTGCAATTCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EcoR I</td>
</tr>
<tr>
<td>AB3</td>
<td>41</td>
<td>69.7</td>
<td>GCCGATGATCGATCGATCGCTGCCAGAGCGCCATGAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nco I</td>
</tr>
<tr>
<td>AB4</td>
<td>29</td>
<td>61.3</td>
<td>AATTGCGCCATGCGCTGAGTAGGCCAAA</td>
</tr>
</tbody>
</table>

Table 2.2: Oligonucleotide sequences used in the production of psPLA\(_2\) in MEL cells. The restriction enzyme sites and the ATG start codon are shown in underlined bold type.

\(^{a}\) The \( T_m \) values were calculated using the following equation: \( T_m = 81.5 + 16.6\log_{10}(I^* \text{m}) + 0.41(\%G+C) - (600I^*\text{m}) - 0.63(\%FA) \) (from Newton and Graham, 1994).

where \( I^* \) = the molar concentration of monovalent cations (in this case 0.05 M); \( I \) = oligonucleotide length and FA = formamide (which was 0).

![Diagram](EcoRI AB1 5' AB2 3' 3' AB3 5' NcoI 3' AB4 5')

**Fig 2.3:** Oligonucleotides used in the production of psPLA\(_2\) in MEL cells. The figure shows a schematic representation of the orientation of the annealed oligonucleotides.
30 pmoles of each of the oligonucleotides were treated with T4 polynucleotide kinase (Appendix A2.4), extracted with phenol chloroform and precipitated with isopropanol. The oligonucleotides were dissolved in sterile water to a final concentration of 0.3 µM. 0.3 pmoles of each of the oligonucleotides and 0.3 pmoles of psPLA\textsubscript{2}-Bluescript (which had been digested with EcoR I and Sal I) were mixed and heated to 65°C for 1 minute and then cooled on ice. T4 DNA ligase was then added and incubated overnight at 16°C (Appendix A2.5). A PCR was carried out using two external primers (0.2 µM of each), AB1 as the 5' primer and a universal primer which annealed to Bluescript downstream of the psPLA\textsubscript{2} cDNA. The PCR cycling conditions used were: denaturation 1 minute (92°C); annealing 30 seconds (55°C); and primer extension for 1 minute (72°C) for 25 cycles. The ~500 bp PCR product was purified by electrophoresis in a 1.5% agarose gel followed by electroelution (Appendix A3.4). The isolated DNA was digested with EcoR I and Sal I and again purified by electrophoresis in a 1.5% agarose gel followed by electroelution. The DNA purified was ligated into the EcoR I/Sal I sites of Bluescript. Competent DH5\textalpha{} E. coli cells were transformed with the product and the cells were plated out onto AXI plates and grown overnight at 37°C. Several white colonies were picked from the plate, grown up in 150 mL of liquid culture and the plasmid DNA isolated using Qiagen-tip-100 columns (Appendix A3.8). Positive clones were identified by cutting the plasmid DNA with Neo I which produced a ~3.5 Kb linear fragment on an agarose gel. The vector was digested using EcoR I and Xho I and the purified DNA fragment cloned into the EcoR I/Xho I sites of pEV3. Both strands of the gene were sequenced using the T\textsuperscript{7} Sequencing kit (Appendix A4.2). The psPLA\textsubscript{2} gene (including the signal sequence) was sequenced in Bluescript and pEV3 using the appropriate primers. Once the gene sequence had been verified, the pEV3-psPLA\textsubscript{2} plasmid DNA was used to transform MEL cells by electroporation (Section 2.3.2).

2.3.2 Transformation of MEL cells by electroporation.

250 mL of LB media containing 100 µg/mL of ampicillin was inoculated with E. coli (DH5\textalpha{}) cells containing the pEV3-psPLA\textsubscript{2} and grown overnight at 37°C in a gyratory shaker. The culture was centrifuged at 8,000 x g for 5 minutes and the plasmid DNA from the isolated
cells purified using the Qiagen maxi preparation protocol. Approximately 100 µg of isolated vector DNA was linearised using Pvu I, isopropanol precipitated and redissolved in 500 µL of Super Q water.

MEL cells were grown to a density of 5 x 10^6 cells/mL in 10 mL of Dulbecco's modified Eagle medium containing 4% foetal calf serum, 60 µg/mL penicillin and 100 µg/mL streptomycin. The cells were centrifuged (5,000 x g for 2 minutes) and washed with 20 mL of phosphate-buffered saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ pH 7.4 with HCl and sterilised by autoclaving). The cells were centrifuged and resuspended in 0.9 mL of electroshock buffer (25 mM HEPES pH 7.15, 140 mM sodium chloride, 0.7 mM Na₂HPO₄). The linearised DNA was incubated for 10 minutes with the cells in a electroporation cuvette, after which a single pulse of 250 V, 960 µF was delivered (time constant 12 ms). The cells were left for 10 minutes, and then diluted into 24 mL of Dulbecco's modified Eagle medium containing 4% foetal calf serum, 60 µg/mL penicillin and 100 µg/mL streptomycin. The cells were plated out into 1 mL wells and incubated at 37°C overnight. The antibiotic G418 was added to each well to a final concentration of 800 µg/mL and the cells incubated at 37°C for 8-10 days. 12 clones were picked from the wells and plated out, in duplicate, into 5 mL of fresh growth medium containing G418 (400 µg/mL). When the cell density had reached 8 x 10^5 cells/mL one well from each culture was induced by the addition of dimethylsulphoxide (DMSO) to a final concentration of 2% (v/v). The other well was left uninduced to be used as a negative control. The cultures were incubated at 37°C for 4 days, after which the cells were centrifuged (10,000 x g for 5 minutes) and positive clones identified using the ¹⁴C-oleate E. coli membrane PLA₂ assay (Section 2.6.12). The supernatant was treated with trypsin, 0.1% w/w of trypsin to sPLA₂ (pH 7 for 10 minutes at 37°C) assuming that the level of expression of the enzyme was similar to that of sPLA₂ from MEL cells obtained from Dr. M. Hollis (i.e. ~1 mg/L). The supernatant was then assayed for PLA₂ activity using the E. coli membrane assay.
Chapter 2: Materials and methods

2.4 Protein expression and purification.

2.4.1 Overexpression of bovine pro-PLA₂ from *E. coli*.

*E. coli* BL21 (DE3) pLysS cells were transformed with pbPLA₂-pET12a and plated out onto LB agar plates containing 50 µg/mL chloramphenicol and 100 µg/mL ampicillin. Chloramphenicol was required to maintain the pLysS plasmid (carrying the T7 lysozyme gene, see Chapter 3, Results Section 3.1), and ampicillin to retain the pET plasmid. After incubation at 37°C overnight, a single colony was picked and transferred to a minimal media (A salts) plate containing chloramphenicol and ampicillin (Appendix A1.2). The plate was incubated at 37°C until colonies had grown (usually two days). This plate was then used to seed several minimal media plates which would be used to inoculate the main culture.

The lawn of bacterial cells were scraped from the plates and resuspended in 20 mL of A salts media with the appropriate antibiotics. This starter culture was then used to inoculate several 2 L baffled flasks each containing 250 mL of minimal media with 50 µg/mL chloramphenicol and 100 µg/mL ampicillin (Appendix A1.3). The flasks were incubated in a gyratory shaker at 300 rpm at 30°C. Once the O.D.₆₀₀ had reached 2.5-2.8 the expression of protein was induced by the addition of 1 M filter-sterilised IPTG to give a final concentration of 500 µM. The cultures were left for a further 12-14 hours after which the cells were harvested by centrifugation at 8,000 x g for 15 minutes. Cell pellets were stored at -20°C to aid cell lysis prior to purification of the protein.

The mutants of bPLA₂ were grown under identical conditions as described above. ¹⁵N isotopically labelled protein was produced by substituting ¹⁴NH₄Cl with ¹⁵NH₄Cl in the growth media.

2.4.2 Isolation of pbPLA₂ inclusion bodies from *E. coli*.

The purification of PLA₂ expressed in *E. coli* was monitored throughout by SDS PAGE (15%) (Appendix A5.3).
Chapter 2: Materials and methods

The frozen cell pellets from a 1 L culture were resuspended in 25 mL of lysis buffer (0.1 M Tris pH 8.0, 10 mM EDTA, 1% Triton X-100) and PMSF (15 mM stock in isopropanol) added to give a final concentration of 0.1 mM. Dnase I (10 mg), lysozyme (10 mg) and 1 M MgCl₂ (final concentration 10 mM) were added to the suspension and it was left to stir at room temperature for 1 hour. The suspension was centrifuged at 12,000 x g for 10 minutes and the supernatant discarded. The pellet (of inclusion body protein) was resuspended in 25 mL of washing buffer (0.1 M Tris pH 8.0, 10 mM EDTA, 1% Triton X-100) and left to stir at room temperature for 1 hour. After centrifugation of the suspension at 12,000 x g for 10 minutes the resulting pellet was dissolved in 20 mL of 8 M urea, 0.3 M Na₂SO₃. 5 mL of 50 mM disodium 2-nitro-5-thiosulphobenzoate (NTSB) was added to the solution and the reaction left to proceed in the dark at room temperature. Care was taken not to expose high concentrations of urea to direct heat or light. This was because the direct action of light and heat on urea is known to cause its breakdown to form cyanate ions which can chemically modify proteins. The production of NTSB is outlined in Appendix A5.1. The extent of the S-sulphonation was followed by measuring the absorbance at 412 nm; the reaction was complete when there was no further increase in absorbance at 412 nm. After centrifugation at 15,000 x g for 30 minutes (at 4°C), the supernatant was dialysed using Spectra/Por dialysis tubing (M₅ cut-off 8 kDa) against distilled water (3 x 5 L) for 12 hours at room temperature, changing the water every 4 hours. The protein was precipitated by dialysis against 0.3% acetic acid for 3 hours and collected by centrifugation at 12,000 x g for 15 minutes. The pellet was washed three times in distilled water by repeated resuspension and centrifugation (12,000 x g for 10 minutes), lyophilised and stored at -20°C until required.

2.4.3 Overexpression of pro-sPLA₂ in E. coli.

Expression of psPLA₂ in E. coli (DE3) pLysS was as for bPLA₂ but with the following alterations. Cells were plated onto LB agar plates with 100 μg/mL of ampicillin, 50 μg/mL chloramphenicol and the lawn of cells used to inoculate the main culture. The growth media used was 2 x YT and the cultures were grown at 37°C until the O.D.₆₀₀ reached 1, after which protein expression was induced by the addition of 1 M IPTG (to give a final concentration of
500 μM). Cultures were then left for a further 4 hours, after which the cells were harvested by centrifugation (8,000 x g for 15 minutes).

2.4.4 Isolation of psPLA₂ inclusion bodies from E. coli.

The isolation of psPLA₂ inclusion bodies was as for pbPLA₂, but with the following modification to the protocol. After cell lysis the pellet (of inclusion body protein) was resuspended in 25 mL of washing buffer (0.1 M Tris pH 8.0, 10 mM EDTA, 0.2% SDS) and left to stir at room temperature for 1 hour. After centrifugation of the suspension at 12,000 x g for 10 minutes the resulting pellet was dissolved in 20 mL of 6 M guanidine-HCl, 0.3 M Na₂SO₃. From this point on, the method used was as previously described for pbPLA₂ (Section 2.4.2).

S-sulphonated psPLA₂ protein was further purified prior to refolding by gel filtration chromatography using a High Load 26/60 Superdex 75 FPLC column. The column was equilibrated with 500 mL of running buffer (8 M urea, 100 mM sodium chloride and 50 mM Tris-HCl pH 8.0). 20 mg of S-sulphonated protein was dissolved in 4 mL of running buffer and particulate material removed by centrifugation (15,000 x g for 15 minutes). After loading the sample, the column was run at a flow rate of 0.2 mL/minute with running buffer and 2.5 mL fractions were collected. Fractions containing psPLA₂ (as judged by 15% SDS-PAGE) were pooled, dialysed against 3 x 5 L of distilled water and precipitated by dialysis against 0.3% glacial acetic acid (v/v). Insoluble protein was then collected and washed by repeated resuspension in distilled water and centrifugation (12,000 x g for 10 minutes) and finally lyophilised.

2.4.5 Expression and purification of MEL cell sPLA₂.

MEL cells which secreted the active form of sPLA₂ (pEV3-sPLA₂) were a gift from Dr. M. Hollis. The MEL cells were grown in Dulbecco's modified Eagle medium containing 4% foetal calf serum, 60 μg/mL penicillin, 100 μg/mL streptomycin and 400 μg/mL G418. Once the cell density had reached 8 x 10⁵ cells/mL the cultures were induced by the addition of 2%
Chapter 2: Materials and methods

DMSO and allowed to grow for a further 4 days at 37°C. The cells were centrifuged (5,000 x g for 5 minutes) and the pH of the supernatant reduced to pH 4.5 by the addition of 1 M acetic acid. The supernatant was loaded on to a Fast Flow S Sepharose cation exchange column previously equilibrated with 50 mM sodium acetate pH 4.5. After applying the sample, the column was washed with 50 mM sodium acetate pH 4.5 until the absorbance returned to baseline. A linear salt gradient of 0-1.6 M sodium chloride in 50 mM sodium acetate pH 4.5 was used to elute the protein from the column. Fractions which contained sPLA₂ activity were pooled and an equal amount of 1.7 M ammonium sulphate added. The protein solution was further purified using a Phenyl Superose 5/5 FPLC column. The column was initially equilibrated with 10 mL of 1.7 M ammonium sulphate, 50 mM sodium acetate pH 4.5, after which the protein was applied. The protein was eluted with a 20 mL linear gradient from 1.7 to 0 M ammonium sulphate at 0.5 mL/minute and fractions collected. Protein containing fractions were assayed for sPLA₂ activity using labelled ¹⁴C E. coli membranes (see Section 2.6.12).

2.5 In vitro refolding and purification of recombinant PLA₂.

2.5.1 Refolding of pro-bPLA₂ in the absence of molecular oxygen.

The apparatus was set up as shown in Fig. 2.4 with 500 mL of denaturing buffer (8 M urea, 25 mM sodium borate pH 8.7, 5 mM potassium EDTA, 2 mM oxidised glutathione [Appendix A5.2], 4 mM reduced glutathione) in the dropping funnel and 3.5 L of refolding buffer (25 mM sodium tetraborate pH 8.7, 2 mM oxidised glutathione, 5 mM potassium EDTA, 4 mM reduced glutathione) in the 5 L round bottom flask. Molecular oxygen was removed by degassing the solutions in situ under vacuum with vigorous stirring and followed by introducing argon gas into the apparatus; this was repeated several times. Approximately 180 mg of S-sulphonated protein was dissolved in 30 mL of denaturing buffer and the mixture stirred vigorously for 1 hour in the absence of light. Insoluble protein was removed by centrifugation (12,000 x g for 20 minutes at 4°C). The S-sulphonated protein solution was carefully added to the denaturing buffer held in the dropping funnel and the solutions again
Chapter 2: Materials and methods

degassed in situ. The 500 mL of denatured protein solution was added dropwise to the refolding buffer over 2-4 hours in the absence of light (achieved by covering the apparatus with a large black plastic bag) to give a final protein concentration of 45 mg/L. During this period the refolding buffer was stirred gently and argon gas was continuously passed through the apparatus to maintain an oxygen free environment. The refolding solution was left stirring for a further 18 hours with the exclusion of light. The pH of the protein solution was decreased to 4.5 using 2 M HCl and any precipitated protein was removed by centrifugation (10,000 x g for 30 minutes). The protein solution was diluted (to 8-10 L) until the conductivity was reduced to 2-4 mS. A Fast Flow S-Sepharose cation exchange column (with a bed volume of 80 mL) was equilibrated with 300 mL of 20 mM sodium acetate pH 4.5 at 4°C. After loading the protein solution at a flow rate of 15 mL/minute, the column was washed with 200 mL of 20 mM sodium acetate pH 4.5. 200 mL of 1 M sodium chloride, 20 mM sodium acetate pH 4.5 was used to elute the protein from the column and the protein containing fractions dialysed against distilled water (3 x 5 L at room temperature). A reduction in volume was achieved by freeze-drying the dialysed protein solution.

2.5.2 Activation and purification of pro-bPLAg.

The freeze-dried pro-bPLAg (pbPLAg) protein was redissolved into trypsin cleavage buffer (10 mM Tris-HCl pH 8.0 and 10 mM calcium chloride) to give a final protein concentration of 5 mg/mL. The pbPLAg mixture was activated by the addition of 0.05% trypsin (w/w) at room temperature. 10 µg of protein was periodically taken and assayed for activity using the egg-yolk lecithin assay (see Section 2.6.5). Once there was no further increase in bPLAg activity, the activation was terminated by the addition of an equal volume of 2 M ammonium sulphate, 40 mM sodium acetate pH 4.5. Insoluble protein was removed by centrifugation (15,000 x g for 15 minutes) prior to loading the sample onto the FPLC hydrophobic interaction column. The protein was purified using the Pharmacia FPLC system and the appropriate columns (Sections 2.5.3 to 2.5.5).
Fig. 2.4: Apparatus used to refold PLA₂ under argon at room temperature. The apparatus was shielded completely from light during the procedure. The pressure inside the apparatus during degassing was measured using the pressure gauge. Degassing was facilitated by the rapid stirring of the solutions. The S-sulphonated protein was refolded by the drop-wise dilution of the protein from 8 M to 1 M urea over 2-4 hours, after which the protein solution was left to stir for a further 18 hours.
Chapter 2: Materials and methods

2.5.3 Purification of bPLA₂ using hydrophobic interaction chromatography.

Solutions used for hydrophobic interaction chromatography were buffer A: 20 mM sodium acetate pH 4.5, 15% isopropanol and buffer B: 1 M ammonium sulphate, 20 mM sodium acetate pH 4.5. The Phenyl Sepharose High Performance XK 26/10 FPLC column was equilibrated with 150 mL of buffer B. Activated refolded bPLA₂ (50-100 mg protein) was applied to the column, after which the column was washed with 100 mL of the high salt buffer. The protein was eluted by running a 500 mL linear gradient from 50% buffer B to 100% buffer A at a flow rate of 4 mL/minute. Fractions that contained PLA₂ activity (using the egg-yolk lecithin assay; Section 2.6.5), were pooled, dialysed (at room temperature) against 2 x 5 L of 20 mM sodium acetate pH 4.5 and then freeze-dried.

2.5.4 Purification of bPLA₂ using ion exchange chromatography.

Solutions used for ion exchange chromatography were buffer A: 20 mM sodium acetate pH 4.5 and buffer B: 1 M sodium chloride, 20 mM sodium acetate pH 4.5. The lyophilised protein was dissolved in 5 mL of buffer A and filtered through a 0.45 μm Acrodisc filter. The High Load S-Sepharose High Performance XK 26/10 FPLC column was equilibrated with 150 mL buffer A, and the protein loaded onto the column. 100 mL of buffer A was used to wash the column prior to running the linear salt gradient from 0% to 40% B at a flow rate of 4 mL/minute. Protein containing fractions were again assayed for PLA₂ activity.

2.5.5 Purification of recombinant bPLA₂ by chromatofocusing.

Solutions used for chromatofocusing chromatography were buffer A: 75 mM Tris pH 9.3 and buffer B: Polybuffer 96 pH 6.0. Analytical samples taken from fractions with enzyme activity eluted from the High Load S-Sepharose column were run on a Mono P 5/20 FPLC column. This was done to identify which protein fractions contained only pure recombinant PLA₂. 50 μg of protein solution was mixed with 1 mL of buffer A and applied to the column which had been pre-equilibrated with 40 mL of buffer A. The column was washed with 8 mL
of buffer A before 30 mL of buffer B (at a flow rate of 1.25 mL/minute) was passed through
the column creating a pH gradient from 9.3 to 6 and eluting the bound protein. Protein
fractions which contained only one peak in the elution profile were pooled, dialysed against 5
L of distilled water overnight, and finally freeze-dried.

The same procedures was used to isolate the three mutant proteins of bPLA$_2$ i.e.
V63F, N71E and V63FN71E.

2.5.6 *In vitro* protein refolding of pro-sPLA$_2$.

Approximately 20 mg of S-sulphonated pro-sPLA$_2$ (psPLA$_2$) was dissolved in 250
mL of 8 M urea, 50 mM Tris-HCl pH 8.5 and placed in a 2 L round-bottom flask. The urea-
protein solution was diluted by the dropwise addition of 750 mL of 50 mM Tris-HCl pH 8.5,
2 mM oxidised glutathione, 4 mM reduced glutathione and 10 mM calcium chloride, via a 1
litre dropping funnel over a period of 4 hours and then left stirring overnight in the absence of
light. The pH of the protein solution was decreased to 4.5 using 2 M acetic acid and any
precipitated protein was removed by centrifugation (10,000 x g for 30 minutes). A Fast Flow
S-Sepharose cation exchange column with a bed volume of 10 mL was equilibrated with 50
mM sodium acetate pH 4.5 at 4°C. The protein solution was not diluted, but directly applied
to the column at a flow rate of 5 mL/minute. 1 M sodium chloride, 50 mM sodium acetate pH
4.5 was used to elute the protein from the column. Prior to activation using factor X$_s$ the
solution was dialysed against 3 x 5 L of 50 mM Tris-HCl pH 8.0 at room temperature.

2.5.7 Refolding psPLA$_2$ in the absence of molecular oxygen.

The refolding of psPLA$_2$ was similar to pbPLA$_2$ but with the following modifications
to the procedure. 250 mL of denaturing buffer (8 M urea, 50 mM Tris-HCl pH 8.5, 5 mM
potassium EDTA, 2 mM oxidised glutathione, 4 mM reduced glutathione) was present in the
dropping funnel and 750 mL of refolding buffer (50 mM Tris-HCl pH 8.5, 5 mM potassium
EDTA, 2 mM oxidised glutathione, 4 mM reduced glutathione) was present in a 2 L round
bottomed flask. Approximately 20 mg of S-sulphonated protein was dissolved in 20 mL of
denaturing buffer and the mixture stirred vigorously for 1 hour. Insoluble protein was removed by centrifugation (12,000 \times g for 20 minutes at 4°C). The S-sulphonated protein solution was then added to the denaturing buffer held in the dropping funnel and the solutions again degassed in situ. The denaturing buffer was added dropwise to the refolding buffer over 2-4 hours, in the absence of light, to give a final protein concentration of 20 \mu g/mL. The solution was not diluted but directly applied to a Fast Flow S-Sepharose cation exchange column as described previously (Section 2.5.6).

2.5.8 Activation and purification of psPLA2.

Factor X_{A} (at an enzyme to substrate ratio of 1:100, w/w) and sodium chloride (to a final concentration of 100 mM) were added to the refolded protein and the solution incubated overnight at room temperature. An equal volume of 3 M ammonium sulphate, 40 mM sodium acetate pH 4.5 was added and the solution centrifuged to remove any small particles (15,000 \times g for 10 minutes). The protein solution was further purified using a Phenyl Superose 5/5 FPLC column. Solutions used for hydrophobic interaction chromatography were buffer A: 50 mM sodium acetate pH 4.5 and buffer B: 1.7 M ammonium sulphate, 50 mM sodium acetate pH 4.5. The column was initially equilibrated with 10 mL of buffer B, after which the protein was applied. The protein was eluted with a 20 mL linear gradient from 1.7 to 0 M ammonium sulphate at a flow rate of 0.5 mL/minute and fractions collected. Protein containing fractions were assayed for sPLA2 activity using labelled ^{14}C E. coli membranes (see Section 2.6.12).

2.6 Kinetic methods and binding assays.

2.6.1 Protein concentration determination.

The protein concentration of solutions of pPLA2, bPLA2 and its mutants were determined using several methods. From the measured A_{280} the concentration was determined using ε=13,000 M^{-1} cm^{-1}. The protein concentrations were also determined using the Pierce bicinchoninic acid (BCA) protein assay to confirm that the mutations introduced into bPLA2
did not significantly change the values obtained spectrophotometrically. Native bPLA$_2$ (from 0-20 µg of enzyme at 1 µg intervals) was used to construct the standard curve.

The presence of buffer components (such as surfactants and denaturants) can affect the A$_{280}$ measurements used to calculate protein concentrations. Therefore the Bradford protein dye-binding assay (Bradford 1976) was also used to determine protein concentrations when denaturants and detergents were present. The assay involves the binding of Coomassie Brilliant Blue G-250 to the protein which causes a shift in the absorption maximum of the dye from 465 to 595 nm, and it is this increase in A$_{595}$ which is monitored.

Coomassie Brilliant Blue G-250 (Sigma) (100 mg) was dissolved in 50 mL of 95% ethanol. To this solution, 100 mL of 85% (w/v) phosphoric acid was added and the resulting solution was diluted to a final volume of 1 L with Super Q water.

The protein solution was pipetted into a 1 mL disposable cuvette and the volume was adjusted to 100 µL with the appropriate buffer or distilled water. 1 mL of Bradford reagent was added to the cuvette and the contents mixed immediately by inversion and then left to incubate at room temperature for 2 minutes. The A$_{595}$ of the sample was measured against a reagent blank prepared from 100 µL of the appropriate buffer or distilled water and 1 mL of Bradford reagent. From the A$_{595}$ value measured, the amount of protein present was calculated from the standard curve. The standard curve was produced using native bPLA$_2$ (from 0-20 µg of enzyme at 1 µg intervals), and a new curve was created for each batch of Bradford assay reagent made.

2.6.2 CMC determination using ANS fluorescence.

The critical micellar concentration (cmc) of various surfactants was determined using ANS as a fluorescent probe to detect the formation of surfactant micelles (modified from de Vendittis et al., 1981). Experiments were carried out using a thermostatically controlled Perkin-Elmer LS5B Luminescence spectrophotometer. A 1 mM C$_{16}$PN stock solution was prepared by dissolving the surfactant in a known volume of buffer (100 mM sodium chloride, 50 mM calcium chloride, 100 mM sodium acetate pH 6.0). A quartz cuvette containing 2.5 mL of buffer and 50 µM ANS was incubated at 25°C with continuous stirring. The sample
was excited at 355 nm and the fluorescence emission measured at 450 nm. The excitation and emission slit widths were set at 2.5 and 10 nm respectively. C\textsubscript{16}PN was titrated into the cuvette using a stock solution of surfactant (1 mM) and left to incubate for 3 minutes before fluorescence readings were taken. The titration range for C\textsubscript{16}PN was from 0-40 \textmu M. A plot of the increase in fluorescence intensity against the concentration of surfactant produces two lines, the intersection of which gives the cmc value (see Chapter 6, Section 6.1).

Similar procedures were used for the cmc determinations of HEPC, diC\textsubscript{6}PC and diC\textsubscript{8}PC but with the following modifications. For HEPC, the cuvette was incubated at 37°C and contained 25 mM sodium chloride, 25 mM calcium chloride, 100 mM Tris-HCl pH 8.0. The titration range for HEPC was 0-16 \textmu M. For diC\textsubscript{6}PC and diC\textsubscript{8}PC the cuvette was incubated at 45°C and contained 100 mM sodium chloride, 25 mM calcium chloride, 1 mM sodium tetraborate pH 8.0 as the assay buffer. The titration range for diC\textsubscript{6}PC and diC\textsubscript{8}PC was 0-10 mM and 0-0.6 mM phospholipid respectively. The stock phospholipid solutions (40 mM diC\textsubscript{6}PC and 10 mM diC\textsubscript{8}PC) contained all the components of the assay buffer except for the calcium chloride as its presence caused the aggregation of the amphiphiles to form higher ordered structures.

2.6.3 Fluorescence binding studies of PLA\textsubscript{2} to lysophospholipid analogues.

The interaction of PLA\textsubscript{2} with micelles of lysophospholipid analogues was analysed by fluorescence spectroscopy. C\textsubscript{16}PN stock solutions were prepared by dissolving the surfactant in a known volume of buffer (100 mM sodium chloride, 50 mM calcium chloride, 100 mM sodium acetate pH 6.0). A quartz cuvette containing 2 mL of buffer and 5 \textmu M PLA\textsubscript{2} was incubated at 25°C with continuous stirring. A second cuvette containing buffer only was used as a control to measure the fluorescence of C\textsubscript{16}PN alone. The samples were excited at 295 nm and the fluorescence emission measured at 340 nm. The excitation and emission slit widths were both set at 5 nm. C\textsubscript{16}PN was titrated into both cuvettes and after each addition the sample was left to equilibrate for 3 minutes before the fluorescence was measured. The titration was complete when there was no further increase in the fluorescence of PLA\textsubscript{2}. The
Chapter 2: Materials and methods

observed fluorescence was corrected for the effects of the fluorescence, any inner filter effects from the C16PN itself (Eqn. 2.1) and the increase in volume of the sample (Eqn. 2.2).

\[ F_b = F_t - F_s \]  
(Eqn. 2.1)

Where \( F_b \) = fluorescence corrected for the surfactant control  
\( F_t \) = observed fluorescence  
\( F_s \) = fluorescence of the surfactant control

\[ F_c = F_b \times \frac{V_T}{V_I} \]  
(Eqn. 2.2)

Where \( F_c \) = fluorescence corrected for the dilution effect  
\( V_T \) = total volume of sample  
\( V_I \) = initial volume of sample

The interaction between the protein and micelle (\( F_c \)) was expressed as a fraction of the initial fluorescence of the PLA2 sample in the absence of surfactant (\( F_0 \)) i.e. \( F_c/F_0 \). Thus the relative fluorescence increase \( (F_c/F_0) \) was plotted against the concentration of C16PN. The data were fitted to the Michaelis-Menten equation by non-linear regression using the Grafit software package (Leatherbarrow, 1990) to give the apparent \( K_m \) for micelles of C16PN. The experiment was repeated for pPLA2 and for each of the mutants of bPLA2.

2.6.4 Kinetic studies of PLA2 using the pH-stat.

The kinetic characteristics of bPLA2, pPLA2, V63F, N71E and V63FN71E were investigated using various phospholipid substrates. All experiments were conducted using a pH titration system which consisted of a Radiometer PHM82 standard pH meter, a TTT80 titrator, a ABU80 autoburette and REC80 servograph. A water-jacketed vessel was used to maintain a constant temperature and reaction volumes consisted of 2-4 mL of assay solution. The dilute sodium hydroxide (3-10 mM) solution used to titrate against the reaction mixture was produced by the dilution of standard 97.5 mM sodium hydroxide solution (from Aldrich). Protein stock solutions for kinetic analysis ranged from 0.001-1 mg/mL of PLA2 in distilled
Chapter 2: Materials and methods

water. For all assays (except the egg-lecithin assay) argon gas was passed over the reaction solution (flow rate of 60 mL/minute) to prevent the absorption of atmospheric carbon dioxide.

The 1 M calcium chloride solution had previously been adjusted to pH 8 using dilute sodium hydroxide. For all assays the background rate of carbon dioxide absorption was measured for 2 minutes, after which the reaction was initiated by the addition of \( \text{PLA}_2 \). The initial velocity was measured by recording the amount of dilute sodium hydroxide needed to maintain a constant pH of 8.0 over a given time period. After each assay the reaction vessel was washed several times with distilled water and wiped dry. Using the initial rate measurement the specific activity was calculated using the formula below and expressed as \( \mu \)moles sodium hydroxide added/min/mg enzyme. Since the amount of sodium hydroxide added = the amount of protons liberated by \( \text{PLA}_2 \), the specific activity can also be represented as \( \mu \)moles of \( H^+ \) produced/min/mg enzyme.

\[
\frac{\mu \text{moles of NaOH}}{\text{added/minute}} = \frac{\text{Amount of titrant (\mu moles)} \times \text{Volume of titrant added (uL/minute)}}{1 \times 10^6}
\]  

(Eqn. 2.3)

\[
\text{Specific activity} = \frac{\mu \text{moles NaOH added/minute}}{\text{(\mu moles } H^+/\text{min/mg)}} \times \text{amount of protein (mg)}
\]  

(Eqn. 2.4)

2.6.5 Assays with egg-yolk lecithin/deoxycholate mixed micelles.

The egg-yolk lecithin/deoxycholate mixed micelle assay was similar to the protocol described by Nieuwenhuizen et al., (1974). To the yolk of one egg, 97.5 mL of distilled water and 2.5 mL of 1 M calcium chloride (25 mM calcium chloride final concentration) was added and the solution mixed rapidly. The pH of the mixture was adjusted to 7.95 using 100 mM sodium hydroxide. Mixed micelles were produced by mixing 6 mM deoxycholic acid with the egg mixture in a 2:1 ratio (by volume). 2 mL of the mixed micelle solution was used in each assay and the pH adjusted to 8.0 with titrant (10 mM sodium hydroxide). The reaction was initiated by the addition of \( \text{PLA}_2 \) (1-2 \( \mu \)g) and the rate of addition of sodium hydroxide over a further 4 minutes was measured at 37°C.
2.6.6 \( K_m^{pp} \) determination for micelles of diC\(_2\)PC.

The assay was carried out as described by Noel \textit{et al.}, (1991), but with the following modifications. A 10 mM stock solution of the phospholipid was made by solubilising the lipid in standard assay buffer (100 mM sodium chloride, 1 mM sodium tetraborate pH 8.0) and incubating at 45°C until the solution became clear (~5 minutes). Assays was carried out at 45°C using 5 mM sodium hydroxide as the titrant. The diC\(_2\)PC solution was added to the assay buffer to give the desired phospholipid concentration in a final volume of 1.95 mL. 50 \( \mu \)L of 1 M calcium chloride was added to give a final concentration of 25 mM. The solution was allowed to equilibrate at 45°C and the background rate measured, after which the reaction was initiated by the addition of 0.2 \( \mu \)g (15 pmoles) of PLA\(_2\) and monitored for a further 5 minutes. The concentration range used to determine the \( K_m^{pp} \) for micelles was 0.2-5 mM diC\(_2\)PC. The data were fitted to the Michaelis-Menten equation by non-linear regression using the Grafit software package (Leatherbarrow, 1990) to give the \( K_m^{pp} \) for micelles of diC\(_2\)PC.

2.6.7 \( K_m \) determination for monomeric diC\(_2\)PC.

The same experimental conditions and procedures used for the diC\(_2\)PC assay were used with diC\(_2\)PC (Noel \textit{et al.}, 1991). A stock solution of diC\(_2\)PC was made by drying under vacuum a solution of 100 mg of phospholipid in 5 mL of chloroform. The phospholipid was then solubilised in a known volume of standard assay buffer to give a 40 mM solution of diC\(_2\)PC and incubated at 45°C until the solution became clear. The amount of protein used in each assay was 20 \( \mu \)g (1.5 nmoles) except for the N71E mutant, where 40 \( \mu \)g was used. After the addition of protein, the reaction was monitored for 10 minutes; the concentration range used to determine the \( K_m \) for monomer was 0.25-6 mM diC\(_2\)PC. The data were fitted to the Michaelis-Menten equation by non-linear regression using the Grafit software package (Leatherbarrow, 1990) to give the \( K_m \) for diC\(_2\)PC monomers.
2.6.8 Kinetics on small sonicated vesicles of DMPM.

The experimental conditions under which the DMPM assay was carried out (i.e. high calcium concentration), were such that vesicle fusion was occurring (Jain et al., 1986b; Berg et al., 1991). A 10 mg/mL stock solution of DMPM was prepared by adding the lipid to distilled water (pH 8 with 10 mM sodium hydroxide) and sonicating the solution for 45 seconds. Assays were carried out at 24°C using 3 mM sodium hydroxide as the titrant. The 2 mL assay volume contained 1 mM sodium chloride and 0.02 μg (1.5 pmoles) of PLA2. The pH of the solution was increased to pH 8 using 3 mM sodium hydroxide after which 0.5 mg of DMPM was added. After measuring the background rate, the assay was initiated by the addition of 10 μL of 1 M calcium chloride (final concentration of 5 mM). The reaction was followed for a further 10 minutes and the specific activity calculated as outlined in Section 2.6.4.

2.6.9 Mixed micelles of diC_{14}PC and sodium deoxycholate.

The activity on zwitterionic diC_{14}PC was accomplished by dispersing the substrate with sodium deoxycholate to give mixed micelles. diC_{14}PC on its own forms bilayered structures such as vesicles and PLA2 displays a long lag phase unless an anionic detergent is present. Therefore, mixed micelles were prepared as described by Noel et al., (1991).

Mixed micelles of diC_{14}PC and sodium deoxycholate were prepared by mixing the phospholipid in chloroform and the deoxycholate in methanol at a molar ratio of 1:3. After thorough drying under vacuum, the residue was dissolved in a known volume of standard assay buffer to give a final concentration of 5 mM phospholipid and 15 mM deoxycholate. The solution was brought up to 45°C and pH 8 and incubated at this temperature until it became clear. 5 mM sodium hydroxide was used as the titrant. Calcium chloride was added to
give a final concentration of 5 mM in the 2 mL assay and the kinetic run started by the
addition of 0.5 µg PLA₂.

The above procedure was also used in the preparation and kinetic studies using mixed
micelles of diC₄PG and diC₄PE. Since PLA₂ does not follow Michaelis-Menten kinetics on
mixed micelles only specific activities were measured.

2.6.10 U.V. spectrophotometric assay using micellar HEPC.

The HEPC method used was based on the published procedure by Bhat et al., (1993a),
with several modifications. An U.V. spectrophotometric microtitreplate assay was used to
investigate the kinetic properties of PLA₂ and its mutants on micelles of the synthetic thio-
phospholipid HEPC. Each assay contained 100 µL of 50 mM sodium chloride, 50 mM
calcium chloride, 200 mM Tris-HCl pH 8.0, 50 µL of 10 mM DTNB (made up in ethanol)
and 0.2 µg of PLA₂. The assay volume was kept to 200 µL while varying the concentration of
HEPC and volume of distilled water. The plate was placed in a thermostatically controlled
Labsystems GENESIS microtitreplate reader at 37°C and the absorbance at 405 nm measured
at 20 second intervals over 20 minutes. The assay was linear for up to ~15 minutes and
therefore the absorbance change and hence the initial rate was measured during this time
interval. The substrate concentration range used for the HEPC Kₘ⁺PP determination was 0-6
mM. The amount of protein used in the assay was dependent upon the kₐₚₚ of the enzyme, for
example with the bPLA₂ mutant N71E, 5 µg of protein (instead of 0.2 µg) was used due to the
low catalytic activity of this mutant. The data were fitted to the Michaelis-Menten equation by
non-linear regression using the Grafit software package (Leatherbarrow, 1990) to give the
Kₘ⁺PP for HEPC micelles.

A standard curve was constructed to convert the measured absorbance to a known
concentration of free thiols. This was achieved using 2 mM β-mercaptoethanol as the stock
solution and diluting it to give the concentration range of 0-20 nmoles in a microtitreplate.
Thus by measuring the A₄05 and plotting the data, the relationship between the absorbance
and concentration of free thiols could be determined.
2.6.11 Labelling of E. coli membranes with (1-14C)-oleic acid.

The labelling of the E. coli membranes was modified from the procedure outline by Patriarca et al., (1972). E. coli cells were grown on a modified M9 minimal media plate [with 0.5% glucose (w/v), 0.2% Tween-20 (v/v) and 0.2% oleic acid (v/v)] at 37°C for 48 hours (Appendix A1.1 and A1.2). The cells were then used to inoculate 20 mL of modified M9 broth and grown at 37°C overnight in a gyratory shaker at 230 rpm. The culture was then diluted into 200 mL of modified M9 media and incubated at 37°C for a further 2 hours. The E. coli cells were centrifuged (8,000 x g for 5 minutes) and resuspended in 25 mL of M9 media containing no carbon source. 500 µL of (1-14C)-oleic acid (Amersham 1.96 GBq/mmol, 3.7 MBq/mL) was dried under a stream of nitrogen gas to remove the toluene and then redissolved in 2 mL of 100 mg/ml of fatty acid free-BSA (FAF-BSA) (Chen, 1967). The BSA containing 14C oleic acid was then added to the culture and incubated at 37°C overnight. After the incubation period the culture was centrifuged, the pellet resuspended in 50 mL of 100 mM Tris-HCl pH 8.0, 10 mM calcium chloride and then autoclaved for 10 minutes. Once cooled, a further 150 mL of 100 mM Tris-HCl pH 8.0, 10 mM calcium chloride containing 20 mg/mL of FAF-BSA was added and the suspension mixed thoroughly. The suspension was centrifuged and the supernatant discarded. The pellet was washed with 50 mL of 100 mM Tris-HCl pH 8.0, 10 mM calcium chloride containing 1 mg/mL of FAF-BSA and centrifuged again. The pellet was resuspended in 25 mL 100 mM Tris-HCl pH 8.0, 10 mM calcium chloride containing 1 mg/mL of FAF-BSA. The suspension was divided into 2.5 mL aliquots and stored at -20°C. 100 µL of membranes gave ~ 25,500 cpm (224 Mbq per mole of E. coli spheroplasts). The phospholipid composition of E. coli is 65% phosphatidylethanolamine, 18% phosphatidylglycerol and 12% diphosphatidylglycerol (Evans and Graham, 1991), and thus it was assumed that 100 µL of membranes = 1.9 µmoles of phospholipid.

2.6.12 E. coli membrane assay.

The E. coli membrane assay protocol was modified from the method described by Patriarca et al., (1972). 20 µL of the enzyme solution (0.1-1 µg of protein) was used in each
Chapter 2: Materials and methods

assay; bPLA$_2$ (200 ng) was used as the positive control. The reaction was initiated by the addition of 100 µL of *E. coli* membranes, the suspension vortexed and then incubated at 37°C for 15 minutes. The reaction was terminated by the addition of 20 µL of 2.5 M HCl. Fatty acid liberated during the assay was extracted by the addition of 60 µL of fatty acid free BSA (33.3 mg/mL). The suspension was vortexed and centrifuged at 13,000 rpm for 10 minutes. 200 µL of the supernatant was added to 3 mL of scintillant Optiphase Safe and the solution counted for $^{14}$C-fatty acid liberation. Non-specific release of radiolabel from the substrate was monitored by performing control incubations in the absence of added enzyme (for example, 2% DMSO, ammonium sulphate and sodium chloride solutions where applicable). Any released counts from the controls were subtracted from the values obtained from the assays.

2.7 Nmr spectroscopy and electrospray mass spectrometry.

2.7.1 1D $^1$H nmr of bPLA$_2$ with the titration of RLM-1.

All nmr experiments and data analysis were carried out by Dr. P. Kilby (Biological NMR Centre, University of Leicester). Recombinant bPLA$_2$ for nmr spectroscopy contained 0.5 mM protein, 20 mM $d_4$ succinate pH* 6.1, 50 mM calcium chloride, 125 mM sodium chloride and was made up in $^2$H$_2$O. pH* denotes a pH-meter reading uncorrected for the deuterium-isotope effect on the glass electrode. $^1$H nmr spectra were recorded at 600 MHz on a Bruker AMX600 spectrometer. All spectra were recorded at 314 K and the spectra were referenced internally to CaH of Cys-77 at 5.565 ppm. Typical parameters included 512 scans with 8 K of data points, 0.57 sec acquisition time with a relaxation delay of 1.2 seconds and a sweep width of 12.07 ppm. RLM-1 (stock concentration of ~20 mM in $^2$H$_2$O) was titrated in 3 µL (~0.15 mM final concentration) volumes and $^1$H-nmr spectra recorded between additions. The titration was continued until the RLM-1 concentration appeared to saturate the enzyme as judged by the absence of further changes in the $^1$H spectra. Before Fourier transformation the data were zero filled to 16 K complex points and multiplied by a Gaussian window function. Identical experimental procedures were also used with authentic bPLA$_2$ isolated from pancreas.
2.7.2 NMR spectroscopy of the bPLA$_2$ mutant V63F.

1D $^1$H-nmr of the bPLA$_2$ mutant V63F was carried out under the same conditions as outlined above with the exception that the protein concentration was 0.2 mM. A 2D $^1$H-$^1$H total correlation spectrum (TOCSY) was recorded to verify the presence of the V63F mutation.

2.7.3 Electrospray mass spectrometry.

The exact molecular masses of recombinant bPLA$_2$ and its mutants were determined using electrospray mass spectrometry (ESM). ESM was used to (i) confirm the change in mass of the bPLA$_2$ mutants; (ii) determine if the proteins had been chemically modified by the refolding procedure and (iii) ascertain the percentage incorporation of isotopic label into the $^{15}$N recombinant proteins. ESM experiments were carried out by Dr. G. Eaton (Department of Chemistry, University of Leicester) using a Kratos Concept mass spectrometer coupled with a double-focusing sector analyser. Approximately 200 pmoles (2.8 µg) of lyophilised protein was redissolved in 20 µL of solvent (50% methanol, 49% water and 1% acetic acid). 50 pmoles of the protein solution was injected directly into the spectrometer. The initial mass:charge ratio data was transformed using a computer algorithm to give the molecular mass of the protein.
CHAPTER 3
OVEREXPRESSION, REFOLDING AND
PURIFICATION OF HUMAN SECRETORY PLA2.

RESULTS

3.1 Expression and refolding of pro-sPLA2 from E. coli.

The synthetic sPLA2 gene was engineered with a factor Xa protease digestion site preceding the N-terminus of the protein (Fig. 3.1a and b (4)). This allowed the expressed protein to be cleaved with factor Xa. The factor Xa protease was chosen since the amino acid sequence which it recognises (-IEGRXX-) occurs only once within the expressed protein and not at all within sPLA2. Other proteases such as trypsin may degrade the partially folded enzyme due to their less specific nature. Correctly folded sPLA2 is resistant to the action of trypsin, even given the large number of Arg residues present on the surface of the enzyme (Franken et al., 1992). Trypsin may be used to activate the enzyme, but only after the protein has been folded.

A pro-sequence is required because E. coli initiates translation with N-formylmethionine (fMet) which is then processed by a formylase followed by a Met specific aminopeptidase (if required). The function of N-terminal Met removal is not yet understood. The removal of the N-terminal Met is dependent on the second amino acid: Ala, Ser, Gly, Pro, Thr or Val at this position permits the N-terminal Met to be removed, whereas Arg, Asn, Asp, Gln, Glu, Ile, Leu, Lys or Met do not (Neidhardt et al., 1987).

Expression of the mature protein without a pro-sequence would result in the production of sPLA2 with an N-terminal Met (at position -1, Fig. 3.1b (3)) which is catalytically inactive (Di Marco et al., 1992). This is because the N-terminal Asn-1 is involved in a hydrogen-bonding network with the active site of the enzyme. The side chain of Met is much larger and more hydrophobic than that of Asn (-CH2CONH2). Although the Asn residue is still in the correct position, there is no room for the N-terminal Met and its ammonium cation. It is thus
apparently unable to fit within the active site of the enzyme, to allow the N-terminal ammonium cation to become part of the hydrogen bonding network. However, when the designed construct is expressed within the cell the initiation amino acid Met will be at the N-terminus of the pro-sequence. When the pro-sequence is enzymatically removed after refolding, a free N-terminal Asn-1 will remain (Fig. 3.1b (4)).

Fig. 3.1: (a) Gene construct used to express sPLA\(_2\) (as Pro-sPLA\(_2\)) within the cytoplasm of \textit{E. coli}. The Shine-Dalgarno (SD) sequence is the ribosome binding site. (b) The N-terminal amino acid sequences of the various proteins expressed in MEL cells and \textit{E. coli}. The factor X\(_a\) protease cleavage sites (underlined) and the ompT secretory signal (boxed) are also shown.

Pro-sPLA\(_2\) (psPLA\(_2\)) was overexpressed in \textit{E. coli} using a T7-bacteriophage based expression system (Fig. 3.2). The level of recombinant protein production from the pET system is usually greater than that with \textit{E. coli} based expression systems. This is due to the fact that the T7 RNA polymerase is able to synthesise mRNA at a much greater rate compared
to *E. coli* RNA polymerase (the T7 RNA polymerase is ~7 times more processive than *E. coli* RNA polymerase [Makarova *et al.*, 1995; Studier *et al.*, 1990]).

![Diagram](image)

**Fig. 3.2:** Schematic representation of the control of expression from the pET 12a plasmid carrying the pro-sPLA$_2$ gene by the pLysS plasmid, which encodes the T7 lysozyme. (1) The pET 12a plasmid also encodes the lac I repressor protein which is constitutively expressed from the plasmid. The target of the repressor protein is the lacUV5 promoter of the T7 RNA polymerase (T7 RNA pol) gene which is integrated into the *E. coli* cell chromosome. (2) Although the T7 RNA pol gene is repressed by lac I, occasionally the gene is expressed and so the polymerase is free to transcribe the foreign gene carried by the pET plasmid. (3) To prevent expression from the pET plasmid the bacterial cell also contains the pLysS plasmid. The pLysS plasmid encodes T7 lysozyme, which is constitutively expressed, and binds to T7 RNA pol. (4) The T7 lysozyme-polymerase complex is now unable to transcribe the foreign gene from the pET plasmid. (5) Upon addition of IPTG, the repression of the T7 RNA pol gene is lifted and an excess amount of polymerase is produced which then transcribes the gene carried on the pET 12a plasmid. (6) The level of T7 RNA pol produced is far in excess of the levels of T7 lysozyme, and so transcription from the pET plasmid is unaffected. (7) In the case of pro-sPLA$_2$ the mRNA is translated and the protein forms insoluble inclusion bodies within the cell cytoplasm. (8) With pro-bPLA$_2$, the expressed protein also carries an N-terminal signal sequence (ompT) which directs the protein to the periplasmic space, where after translocation it forms insoluble inclusion bodies.

The pET expression system was ideal for the production of PLA$_2$ (especially sPLA$_2$) within *E. coli*. It was found that both sPLA$_2$ and bPLA$_2$ expressed proteins were toxic to the bacterial cells. Vectors in which protein expression was driven by an *E. coli* promoter (e.g. the
Chapter 3: Overexpression, refolding and purification of sPLA₂

*lac* promoter) were found to have "leaky" expression (i.e. a basal amount of protein was transcribed and translated even in the absence of a chemical inducer such as IPTG). This leaky expression caused a significant problem in the manipulation of the sPLA₂ gene, as even this basal level of protein production was enough to kill the cell. This resulted in the selection of sPLA₂ clones which had DNA sequence deletions or rearrangements which were not toxic to the cell (see Section 3.3.2).

The overexpression of toxic proteins in *E. coli* can affect plasmid stability. If the gene for the toxic protein is not tightly regulated then the low levels of protein expression from the vector will kill the cell. Over several generations the original psPLA₂ gene sequence may be lost through DNA rearrangements, the mutant vector sequence will be favoured as it produces a non-toxic protein. This would significantly reduce the level of recombinant protein expressed upon induction with IPTG, as only a small proportion of the cells are carrying the original gene sequence. Therefore to prevent leaky expression of psPLA₂ from the vector, the pET plasmid was transformed into the *E. coli* cell line BL21 (DE3) pLysS. The pLysS plasmid encodes for T7 lysozyme which is constitutively expressed and has two uses. Firstly it binds to any T7 RNA polymerase produced prior to IPTG induction, thus blocking the polymerase’s ability to recognise and initiate transcription from the promoter of the psPLA₂ gene. This ensures the tight regulation of the foreign gene. Secondly, the T7 lysozyme can be used to help lyse the cells: during growth the T7 lysozyme does not affect the cell as it does not have access to the outer cell wall. After harvesting the cells and storage at -20°C, the cells will have become damaged by the formation of ice crystals and so the T7 lysozyme is now able to degrade the outer peptidoglycan cell wall. Thus there is no need for the use of other methods of cell disruption such as the French press or sonication.

The translated protein formed insoluble inclusion bodies within the cell cytoplasm. The purification of psPLA₂ from *E. coli* was followed by SDS-PAGE (Fig. 3.3). From the gel (lanes 2 and 4) it can be seen that the supernatants from the Triton X-100 washes contain very little psPLA₂ protein. Although the molecular weight of PLA₂ is ~14 kDa, the protein runs anomalously on SDS-PAGE with an apparent molecular weight of ~17 kDa. A large proportion of the contaminating proteins have been removed by the Triton washes. The protein is solubilised during S-sulphonation as can be seen from lane 7 on the gel, but a smaller
amount of protein remains insoluble (lane 6). The soluble S-sulphonated protein was then
dialysed to remove the S-sulphonating and denaturing agents, and lyophilised (lane 8). The
protein was further purified by gel filtration chromatography in the presence of 8 M urea (Fig.
3.4 and Fig. 3.5) prior to refolding. Fractions containing pure psPLA₂ were pooled, dialysed
and lyophilised. The amount of protein used for refolding was such that the final enzyme
concentration after refolding was 20 mg/L.

**Fig. 3.3**: 15% SDS-PAGE gel of the isolation of psPLA₂ expressed in *E. coli*. The lanes marked are: (1) Molecular weight markers, (2) Triton X-100 supernatant wash 1, (3) pellet from Triton X-100 wash 1, (4) Triton X-100 supernatant wash 2, (5) pellet from Triton X-100 wash 2, (6) insoluble protein after S-sulphonation, (7) soluble protein after S-sulphonation and (8) 0.3% acetic acid precipitated protein.
Fig. 3.4: Elution profile of psPLA$_2$ from the Superdex 75 prep grade gel filtration column. 40 mg of protein was loaded in 10 mL of running buffer (8 M urea, 100 mM sodium chloride and 50 mM Tris pH 8.0). Pure psPLA$_2$ eluted in fractions 11-16 (see Fig. 3.5).
Numerous methods of refolding the S-sulphonated protein were investigated. In all cases the final protein concentration was kept at 20 mg/L and the refolding buffer used was 2 mM oxidized glutathione, 4 mM reduced glutathione and 50 mM Tris-HCl pH 8.5, unless otherwise stated. The refolding methods included:
Chapter 3: Overexpression, refolding and purification of sPLA₂

- **Dialysis.** The S-sulphonated protein (250 mL volume) was solubilised in 6 M guanidine HCl and dialysed against 3 x 2 L of refolding buffer. This method of refolding should allow the enzyme to fold slowly, and encourage disulphide bond formation/rearrangement. To reduce aggregation during refolding the dialysis was also carried out at 4°C.

- **Rapid dilution.** The S-sulphonated polypeptide was refolded by the rapid dilution of the denatured protein solution (see Chapter 2, Sections 2.5.6 and 2.5.7). Thus the concentration of the urea solution was rapidly reduced upon mixing with the refolding buffer. The effect of molecular oxygen on refolding was also investigated (Chapter 2, Sections 2.5.6 and 2.5.7), but unlike with pbPLA₂ there was no difference in the amount of enzyme isolated after dilution in the presence and absence of oxygen.

- **Removal of the pro-sequence.** Once the psPLA₂ had been refolded and purified by rapid dilution the N-terminal pro-sequence was removed using factor X₄ protease. The sPLA₂ was then allowed to undergo further disulphide bond shuffling in the presence of a detergent (0.1% Triton X-100) or denaturant (1 M guanidine HCl) and refolding buffer for several days at room temperature. Thus it was hoped that after further disulphide bond shuffling a greater proportion of the enzyme would have the "native like" structure.

- **Altering the refolding buffer.** The components of the refolding buffer were also altered to see if this could improve the final amount of correctly refolded enzyme. For example a non-denaturing detergent (0.1% Triton X-100) was added as it was thought that the surfactant may prevent aggregation of the protein due to hydrophobic interactions. The redox couple used to form the disulphide bonds was also varied, instead of using oxidized and reduced glutathione, 5 mM cystine and 10 mM cysteine were tried.

It proved impossible to refold and purify any significant amounts of psPLA₂ expressed from E. coli. Table 3.1 summarises the protein losses from a typical refolding experiment, which involved rapid dilution of the S-sulphonated protein from 8 to 2 M urea in the presence...
Chapter 3: Overexpression, refolding and purification of sPLA\(_2\)

of refolding buffer (50 mM Tris-HCl pH 8.5, 2 mM oxidized glutathione, 4 mM oxidised glutathione and 10 mM calcium chloride) followed by ion exchange chromatography. A single protein was eluted from the ion exchange column, as judged by SDS-PAGE (data not shown). The protein was activated by factor X\(_4\) and finally purified by hydrophobic interaction chromatography (Table 3.2 and Fig. 3.6). The loss of protein during refolding and isolation was attributed to the hydrophobic nature of the enzyme.

<table>
<thead>
<tr>
<th>Experimental procedure</th>
<th>Protein (mg)</th>
<th>Percentage of protein remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubilised protein in 8 M urea.</td>
<td>42.5</td>
<td>100</td>
</tr>
<tr>
<td>Diluted protein solution in 2 M urea.</td>
<td>18.5</td>
<td>43.5</td>
</tr>
<tr>
<td>Elution from S Sepharose column.</td>
<td>3.20</td>
<td>7.5</td>
</tr>
<tr>
<td>Total protein eluted with sPLA(_2) activity from the Phenyl Superose column.</td>
<td>0.34</td>
<td>0.79</td>
</tr>
<tr>
<td>Fraction with the greatest sPLA(_2) activity from the Phenyl Superose column.</td>
<td>0.07</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Table 3.1: Losses of protein during refolding and purification of sPLA\(_2\) overexpressed in E. coli. Protein concentrations were determined using the Bradford assay. The results are from a 2 L refolding and the protein was refolded by rapid dilution (see Chapter 2, Section 2.5.6).

Fig. 3.6 shows the elution profile of sPLA\(_2\) from the Phenyl Superase column. All the protein containing fractions were assayed using the \(^{14}\)C E. coli membrane assay. The E. coli \(^{14}\)C labelled membrane assay is very sensitive, but is dependent on how efficiently the \(^{14}\)C labelled oleate is taken up and incorporated into the cell membrane. Therefore, the relative specific activities measured can only be compared from assays performed using membranes from the same preparation. Only ~6% of the \(^{14}\)C-oleate added was esterified into the E. coli spheroplasts. Oleate has been shown to be exclusively incorporated into the sn-2 position of phospholipids (Patriarca et al., 1972). The approximate specific activity of the labelled membranes was found to be 224 MBq per mole of E. coli spheroplasts (assuming that the phospholipid composition of E. coli is 65% phosphatidylethanolamine, 18% phosphatidylglycerol and 12% diposphatidylglycerol [Evans and Graham, 1991], and that 100
Chapter 3: Overexpression, refolding and purification of sPLA₂

μL of membranes = 1.9 μmoles of phospholipid). It is clear that even though a single protein was applied to the column (as judged by SDS-PAGE, Fig. 3.5 fractions 11-16), the elution profile from the Phenyl Superose column suggests that a large population of protein conformers exists as the protein elutes over a large volume. The activity of the fraction with the greatest sPLA₂ activity is shown in Table 3.2. There appears to be no significant peak in the elution profile which corresponds to the activity measured, suggesting that only a very small proportion of the protein refolded has the correct conformation. The refolded sPLA₂ isolated had ~2.4 % of the activity of MEL cell expressed sPLA₂. The amount of protein which was able to be activated did not increase with the use of trypsin (data not shown), suggesting that factor X₄ was able to remove the pro-sequence effectively.

<table>
<thead>
<tr>
<th>Purification procedure</th>
<th>Amount of protein (mg)</th>
<th>sPLA₂, relative specific activity (μmoles/min/mg)</th>
<th>Total activity (μmoles/min)</th>
<th>Purification</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein eluted from S-Sepharose column and then factor X₄ activated</td>
<td>3.2</td>
<td>0.35</td>
<td>1.12</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Fraction eluted from the Phenyl Superose column with the greatest activity</td>
<td>0.07</td>
<td>6.4</td>
<td>0.448</td>
<td>18.3</td>
<td>40</td>
</tr>
<tr>
<td>Bovine pancreatic PLA₂ control</td>
<td>-</td>
<td>44.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MEL cell sPLA₂</td>
<td>-</td>
<td>263.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.2: Purification of recombinant sPLA₂ (from a 2 L refolding using ~ 42.5 mg of S-sulphonated protein) overexpressed in E. coli. The enzyme activities were measured using the ³¹C E. coli membrane assay (see Chapter 2, Section 2.6.12 for experimental conditions). Purification and yield values were calculated as follows:

\[
\text{Purification} = \frac{\text{Specific activity of protein at stage of isolation}}{\text{Specific activity at the start of the purification}}
\]

\[
\text{Yield} (\%) = \frac{\text{Total activity of protein at stage of isolation} \times 100}{\text{Total activity at the start of the purification}}
\]
3.2 Expression of sPLA$_2$ from MEL cells.

Expression of the sPLA$_2$-cDNA in murine erythroleukaemia (MEL) cells was achieved using the pEV-3 vector. The pEV-3 vector was designed specifically for the high level expression of foreign proteins in erythroid cell lines and relies on the human globin locus control region (LCR) (Needham et al., 1992). The pEV-3 vector allows the integration position-independent expression of heterologous genes in MEL cells. Thus even if the linearised vector integrates within a "transcriptionally silent" region of a chromosome, the presence of the LCR ensures the high level expression of the cloned gene. The level of expression is dependent on the number of copies of the vector that have integrated within the genome (Needham et al., 1992). The function of the LCR in vivo is to regulate transcription of the $\beta$-globin gene cluster during development and is located $\sim$50 kb upstream of the genes (Evans et al., 1990; Crossley and Orkin 1993). MEL cells are erythroid progenitor cells
Chapter 3: Overexpression, refolding and purification of sPLA₂

derived from the spleens of susceptible mice infected with the Friend virus complex. Once transformed, the cells are arrested at the proerythroblast stage and can be maintained indefinitely (Antoniou, 1991). Fig. 3.7 shows the induction pathway for the expression of sPLA₂ from MEL cells.

Fig. 3.7: Schematic representation of the induction pathway for the expression of sPLA₂ from MEL cells. Dimethyl sulphoxide (DMSO) causes the differentiation of the progenitor cells into mature mouse erythroid cells. The onset of differentiation initiates the production of haemoglobin expression which also causes the transcription from the human β-globin promoter located on the pEV-3 vector. The mRNA produced is translated and the expressed protein targeted for secretion into the culture medium.
Chapter 3: Overexpression, refolding and purification of sPLA₂

The sPLA₂-pEV-3 vector transfected into MEL cells and stable clones isolated using G418 sulphate selection, was a gift from Dr. M. Hollis (ICI pharmaceuticals) (construct 1, Fig. 3.8). G418 sulphate is a member of a family of antibiotics known as aminoglycosides and is toxic to prokaryotic and eukaryotic cells. Resistance to G418 sulphate is conferred by the gene encoding aminoglycoside phosphotransferase 3'.

With purified sPLA₂ (also obtained from Dr. M. Hollis), the relative specific activity on ¹⁴C oleate labelled E. coli membranes at 37°C was determined and found to be 263.6 μ moles/min/mg (Fig. 3.9).

Fig. 3.8: Constructs used to express sPLA₂ in MEL cells under the control of the human globin locus control region (LCR). Construct 1 was a gift from Dr. M. Hollis. Construct 2 was produced so that MEL cells would secrete inactive sPLA₂, which could be activated by factor X₃ or trypsin after purification.
Chapter 3: Overexpression, refolding and purification of sPLA₂

![Graph showing activity of sPLA₂ expressed from MEL cells. 130 ng of protein was used in each assay. The results are from duplicate assays at 37°C, the specific activity was calculated from the initial rate.]

**Fig 3.9:** Activity of sPLA₂ expressed from MEL cells. 130 ng of protein was used in each assay. The results are from duplicate assays at 37°C, the specific activity was calculated from the initial rate.

### 3.2.1 Expression of sPLA₂ (construct 1) from MEL cells.

The secreted enzyme was found to give some measurable activity in the supernatant using the *E. coli* membrane assay. The elution profile of sPLA₂ from the S-Sepharose column is shown in Fig. 3.10. From Fig. 3.10 it can be seen that sPLA₂ activity was located at the tail region of the main peak. These fractions were then pooled and the enzyme purified using the Phenyl Superose column (Fig. 3.11), the greatest activity eluted at ~0.8 M ammonium sulphate (Table 3.3). Thus using this purification procedure sPLA₂ can be further purified from the cell culture medium, but since only 1 litre of cells were grown at a time, the amount of enzyme found in the most active fraction was very low (~100 μg, which is a yield of ~20%). The hydrophobic nature of the enzyme may also cause significant losses on glass surfaces during purification (Kramer and Pepinsky, 1991). The level of expression of sPLA₂ from MEL cells has been shown to be relatively low (0.5 mg/L of culture) when compared to the expression of human growth hormone (100 mg/L of culture) after 7 days of induction (Needham *et al.*, 1992). Thus the expression from MEL cells produces very limited amounts...
of enzyme which increases the difficulty of isolating such low amounts of sPLA$_2$ from the large volume of culture medium used.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total volume (mL)</th>
<th>sPLA$_2$ activity from 20 μL of solution (nmoles/min)</th>
<th>Total activity (μmoles/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEL cell culture supernatant</td>
<td>200</td>
<td>2.5</td>
<td>25.39</td>
</tr>
<tr>
<td>Pooled fractions with sPLA$_2$ activity from the S-Sepharose column</td>
<td>160</td>
<td>2.3</td>
<td>18.4</td>
</tr>
<tr>
<td>Pooled fractions with sPLA$_2$ activity from the Phenyl Superose column</td>
<td>3</td>
<td>13.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Bovine pancreatic PLA$_2$ control (0.2 μg)</td>
<td>0.02</td>
<td>6</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.3: Purification of MEL cell sPLA$_2$ (construct 1). The specific activity of the membranes was 140 MBq per mole of *E. coli* spheroplasts (100 μL of membranes = 1.9 μmoles of phospholipid).

![Fig. 3.10: Purification of MEL cell sPLA$_2$ (construct 1). 200 mL of supernatant from the MEL cells was loaded onto the Fast Flow S-Sepharose column. The column was equilibrated with 0.4 M sodium chloride and 50 mM sodium acetate pH 4.5. The protein was eluted with a linear gradient of 0-1.6 M sodium chloride. Fractions were then assayed for sPLA$_2$ activity using the *E. coli* membrane assay.](image-url)
Fig. 3.11: Elution profile of MEL cell sPLA$_2$ (construct 1) using a Phenyl Superose HR 5/5 column. The protein was eluted by the reduction in the concentration of ammonium sulphate. Fractions were assayed for activity using the *E. coli* membrane assay.

### 3.2.2 Construction of pro-sPLA$_2$ (construct 2) for MEL cell expression.

Another reason for the low amount of enzyme isolated (0.5 mg sPLA$_2$/L of culture compared to ~100 mg human growth hormone/L [Needham *et al.*, 1992]), may be due to the fact that sPLA$_2$ is secreted into the culture media. It was speculated that active secreted enzyme may hydrolyse the phospholipids present in the cell membranes, thus lysing the cells. Therefore after protein induction, the MEL cells would gradually be destroyed by the action of the secreted enzyme, thus reducing the number of cells which were able to produce sPLA$_2$. Hence construct 2 was made (Fig. 3.8), in which the human sPLA$_2$ secretory signal and a Kozak sequence were cloned upstream of factor X$_a$-sPLA$_2$ (pro-sPLA$_2$). A factor X$_a$ sequence was engineered upstream of the sPLA$_2$ cDNA by Dr. P. Mallinder. The Kozak sequence is a eukaryotic ribosome initiation site with the consensus sequence CCN/gCCATGG as identified by single base substitutions around the ATG initiator codon in a cloned preproinsulin gene.
Chapter 3: Overexpression, refolding and purification of \( sPLA_2 \)

(Kozak, 1986a; 1986b). Therefore the secreted enzyme would be inactivated by the N-terminal extension which could then be enzymatically removed in vitro by factor Xa or trypsin. It was therefore hoped that the secretion of the inactive enzyme would increase the final amount of protein isolated, as the cells would not be lysed during the period of psPLA\(_2\) expression.

The construction of pro-\( sPLA_2 \) (psPLA\(_2\)) in pEV3 for expression in MEL cells was hampered by base deletions within the gene during manipulations in the cloning vector pBluescriptII SK (pBSII SK). The pBluescript system uses \( \beta \)-galactosidase to discriminate between bacterial colonies carrying re-circularised vector DNA from those carrying vector-insert DNA. Cells carrying the pBSII SK-insert DNA were plated out onto LB agar containing X-gal and IPTG. IPTG causes the continual expression of \( \beta \)-galactosidase from the lac promoter. X-gal, when hydrolysed by \( \beta \)-galactosidase, produces a chromogenic compound which colours the growing colonies blue. If the DNA insert is present, the \( \beta \)-galactosidase gene will be disrupted (as it contains the multiple cloning site), and thus the cells continually express a \( \beta \)-galactosidase-fusion protein. This fusion protein is unable to hydrolyse X-gal and so the colonies remain white.

The frequency of the rate of mutation in \( E. coli \) is between \( 10^{-7} \) to \( 10^{-11} \) per replication event (Watson, \textit{et al.}, 1990). Positive psPLA\(_2\) clones (as identified by the unique \textit{Nco} I site) were sequenced and all contained at least one base deletion which is a much higher mutation rate than might be expected in \( E. coli \). A possible reason for this high mutation rate was attributed to the \( \beta \)-galactosidase-fusion protein being toxic to the cells. The only viable cells are those in which a deletion mutation has arisen which will cause a codon frame shift. The DNA frame shift produces a protein with a totally different peptide sequence which is nontoxic to the cell. Thus only cells carrying the psPLA\(_2\) gene with a mutation will grow and be detectable with the blue/white selection method. To overcome this problem the manipulations of psPLA\(_2\) were carried out in pBluescript KS. The cloning site in KS is orientated in the opposite direction compared to SK. Thus the cloned insert will be in the opposite orientation and the \( \beta \)-galactosidase-fusion protein produced should no longer be toxic since the psPLA\(_2\) gene will produce a nonsense protein in this orientation. Using this method all subsequent clones isolated contained the correct DNA sequence for psPLA\(_2\).

93
Chapter 3: Overexpression, refolding and purification of \textit{sPLA}_2

Construct 2 was made, but was not transfected into MEL cells for two reasons. Firstly it came to light that \textit{psPLA}_2 would not fold correctly when overexpressed in methylotrophic yeast (Section 3.3). Since both yeast and MEL cells have similar mechanisms of protein folding and translocation, this suggested that \textit{psPLA}_2 would also behave in a similar manner when overexpressed in MEL cells. Secondly, it was discovered that active \textit{sPLA}_2 is unable to hydrolyse the phospholipid membranes of yeast or \textit{E. coli} unless they have been autoclaved (Harris et al., 1991). Therefore the continuation and completion of the expression of \textit{psPLA}_2 from MEL cells would not improve the levels of protein isolated.

3.3 Expression of \textit{psPLA}_2 from methylotrophic yeast.

A yeast expression system was also used to express \textit{sPLA}_2 by secreting the protein into the growth media. The expression of \textit{sPLA}_2 from methylotrophic yeast should produce more protein than the MEL cell expression system due to the very high cell density which can be achieved with yeast cells. Methylotrophic yeast (\textit{Hansenula polymorpha}) can be grown to high cell densities (100 g dry weight per litre) and can accumulate ~1 g of recombinant protein per litre of culture broth (for example glucoamylase 1.4 g/litre of cell culture, Gellissen et al., 1992). This high level of protein production is achieved by inducing the expression with methanol. Methanol is used as the sole carbon source for growth and this causes the induction of enzymes (such as formate dehydrogenase, FMD) responsible for methanol metabolism. The methanol inducible enzymes have strong promoters which have been used to drive the expression of the cloned DNA in the vector pFMD13025. The pro-\textit{sPLA}_2 gene was cloned into this vector by Dr. P. Mallinder and the cultures grown by Dr. R. Badii (Department of Biochemistry, University of Leicester). The growth media which contained the secreted \textit{psPLA}_2 was concentrated and activated using trypsin. A small amount of \textit{PLA}_2 activity was observed using the \textit{E. coli} membrane assay (Fig. 3.12). The amount of protein produced can be back calculated from the specific activity shown in Fig. 3.8, as the same labelled membrane preparation was used to measure the MEL cell and yeast expressed \textit{sPLA}_2 activity. From the measured \textit{sPLA}_2 specific activity the amount of protein secreted was estimated to be ~10 ng \textit{sPLA}_2/L of culture.
Transmission electron micrographs of the cells (Fig. 3.13a and 3.13b) show a build up of protein at or in the endoplasmic reticulum (Fig. 3.13b). This suggests that the protein either cannot translocate across the endoplasmic reticulum membrane or cannot fold within the endoplasmic reticulum.

**Fig. 3.12:** $^{14}$C *E. coli* membrane assay of secreted pro-sPLA$_2$ from methylotrophic yeast. The yeast culture supernatant was activated using 0.1% trypsin (w/w), after which a protein sample (20 µL) was incubated with the $^{14}$C labelled membranes.

**Fig. 3.13a:** Electron micrograph of methylotrophic yeast (*Hansenula polymorpha*) grown on methanol. The presence of methanol induces the formation of peroxisomes.
Chapter 3: Overexpression, refolding and purification of \( \text{sPLA}_2 \).

Fig. 3.13b: Electron micrograph of \( H. \text{polymorpha} \) carrying the chromosome integrated plasmid encoding \( \text{psPLA}_2 \). Protein expression has been induced with methanol and as can be seen has caused protein aggregation to occur within the cell. The average diameter of the yeast cell is 10 \( \mu \text{m} \).

3.4 Secretion of \( \text{sPLA}_2 \) into the periplasmic space of \( E. \text{coli} \).

From the work on \( \text{psPLA}_2 \) in yeast and \( E. \text{coli} \) cells (vide supra) as well as previous work (Marco et al., 1992; Kelley et al., 1992), it seemed that a free N-terminus may be crucial in protein folding. To achieve this, the secretory signal of the \( E. \text{coli} \) outer membrane protease T (omp T) was placed at the N-terminus of the \( \text{sPLA}_2 \) synthetic gene by Dr. P. Mallinder. The translocation of the protein into the periplasmic space would lead to the removal of the signal sequence leaving Asn at position 1 of the mature protein. Fig. 3.14 shows that after induction with IPTG a band appears at \( \sim 20 \) kDa (lane 2), but there is no band which runs \( \sim 17 \) kDa as determined against authentic \( \text{psPLA}_2 \). The protein was further purified to identify if even small quantities of protein had been translocated. It appears that the protein has not been processed by the secretory machinery into the periplasmic space.
Chapter 3: Overexpression, refolding and purification of sPLA$_2$.

Fig. 3.14: 15% SDS-PAGE gel of the isolation of OmpT-sPLA$_2$ expressed in *E. coli* cultured on A salts minimal media at 30°C. The lanes marked are: (1) Non-induced cells, (2) 0.5 mM IPTG induced cells, (3) Triton X-100 supernatant wash 1, (4) pellet from the Triton X-100 supernatant 1, (5) soluble protein after S-sulphonation, (6) insoluble protein after S-sulphonation, and (7) S-sulphonated pro-sPLA$_2$. 
Chapter 3: Overexpression, refolding and purification of sPLA₂.

DISCUSSION

3.5 *E. coli* derived recombinant sPLA₂.

Nmr experiments require relatively large quantities of purified sPLA₂. Expression of sPLA₂ from eukaryotic cells has been shown to be very limited in terms of the quantities of enzyme that can be produced, for example ~400 μg/L of protein is secreted from Chinese hamster ovary cells (Johansen *et al.*, 1992). Thus the high level of protein expression, the well known genetic characteristics, and the ease of culturing cells makes *E. coli* the organism of choice for the recombinant expression of sPLA₂. The major problem with the prokaryotic system is that the cells lack the machinery to efficiently fold disulphide bond containing proteins within the cytoplasm. Disulphide bond formation in *E. coli* occurs within the periplasmic space, where dedicated enzyme systems exist (Wülfing and Plückthun, 1994; Bardwell, 1994).

The *in vitro* refolding and isolation of psPLA₂ was hampered by the intrinsic insolubility of the inclusion body protein which was only partially soluble in 8 M urea or guanidine-HCl. A large proportion of the protein precipitated out of solution during the refolding procedure, this increased the difficulty of isolating the protein as the enzyme solutions were so dilute. Also, the hydrophobic nature of the protein significantly reduced the final amount of enzyme isolated due to losses on/at glass surfaces (Kramer and Pepinsky, 1991). The folding of psPLA₂ under various conditions produces protein with a very low specific activity (~2.6% when compared to purified MEL cell sPLA₂). This may be caused by the incorrect folding of the enzyme due to the presence of the pro-sequence. Initial experiments in the folding of porcine PLA₂ by Franken *et al* as a *cro-lacZ* fusion protein produced 6 mg per litre of recombinant protein after trypsin activation of the fusion protein. A similar method was then tried with sPLA₂, but an Arg was placed in front of the PLA₂ polypeptide so that after folding the protein could be released from the fusion by trypsin. It was found that trypsin digestion did not produce any sPLA₂ and actually digested psPLA₂ to small peptides, so it was concluded that the protein cannot fold correctly with a N-terminal
Chapter 3: Overexpression, refolding and purification of sPLA₂

fused protein. PLA₂ is normally very resistant to trypsin cleavage (Franken et al. 1992), even though there are a large number of Arg residues present on the surface of the enzyme. This has been attributed to the compact nature of the protein. Therefore if the folded sPLA₂ is being degraded it suggests that the protein does not have the correct fold due to incorrect disulphide bridge formation.

The most successful method of producing sPLA₂ with native structure so far has been the cro-lacZ fusion strategy developed by Franken et al., (1992). Cyanogen bromide (CNBr) was used to cleave the protein after a Met residue (which had been introduced at position -1 of the amino acid sequence), and thus release the protein from the fusion before folding. sPLA₂ also contains a Met at position 8 but this residue is not highly conserved (20% of PLA₂S contain a Leu at this position). Thus Met-8 was changed to Leu-8 to prevent internal cleavage of sPLA₂. To see if Met-8 had any functional role on binding or activity in PLA₂ the double mutant of porcine pancreatic PLA₂ was also constructed by Franken and coworkers (both Met-8 and Met-20 were substituted with Leu). It was shown that the protein folded to produce an enzyme which had nearly identical binding and kinetic properties with various substrates compared to native porcine PLA₂. The porcine fusion protein was cleaved with CNBr before and after folding of the protein to determine if the N-terminal domain interfered with folding. For porcine PLA₂ the N-terminal domain did not interfere with folding (as was also observed using trypsin cleavage of cro-lacZ porcine PLA₂). When repeated for sPLA₂ it was found that removal of the cro-lacZ domain before folding was essential for the enzyme to have any catalytic activity (Franken et al., 1992).

Di Marco et al showed that sPLA₂ with an extra amino acid before the N-terminal Asn (see Fig. 3.1) allows the protein to fold correctly, but the resultant enzyme had only 1% of the activity of sPLA₂ isolated from synovial fluid (Di Marco et al., 1992). This reduction in activity was attributed to the new N-terminal ammonium cation having a different orientation and thus not being able to interact in the same way with the water molecule that, in the native enzyme, links the N-terminus and the side chain of Asp-99.

The Southampton group (Dr. D. Wilton, personal communication) have found that substitution of Asn-1 with Ala allows the N-terminal Met (at position -1) on the expressed polypeptide to be removed by E. coli. This mutant folds and has similar activity to native
Chapter 3: Overexpression, refolding and purification of sPLA₂

sPLA₂ but there are problems with the isolation of the enzyme due to the hydrophobic nature of the protein. The amino acid at position one is vital for PLA₂ catalytic activity at the lipid-water interface; the substituted amino acid has to be able to form the correct hydrogen-bond network with the active site water molecule, Asp-99 and Tyr-52. Therefore Ala was used since it is the N-terminal amino acid in bPLA₂ and hence should make the hydrogen bonding network to the active site.

The PSPLA₂ construct described herein (Mallinder et al., unpublished work) may cause a problem during the refolding of the S-sulphonated polypeptide. The length of the pro-sequence in psPLA₂ (i.e. 5 amino acids including the initiator of translation Met, see Fig. 3.1) may just be long enough to interfere with the folding of sPLA₂ producing a heterogeneous population of molecules, only a small percentage of which have the correct disulphide bridge pattern. As discussed previously, porcine and bovine PLA₂ seem to be able to fold correctly in the presence of N-terminal extensions, even one as large as the cro-lacZ domain (54 kDa). Therefore it may be possible that the secreted digestive pancreatic enzymes, which have a 7 amino acid pro-sequence in vivo which is removed by trypsin to activate the enzyme, have evolved to be able to fold with this N-terminal extension. sPLA₂ does not have a pro-sequence during folding; the protein is expressed with a N-terminus signal sequence in vivo to direct its transport to the endoplasmic reticulum, but this is removed during translocation by a signal sequence peptidase. Thus the 5 amino acid extension to the N-terminus may be enough to interfere with folding in vitro and also, as seen in the expression of the protein from yeast, could cause problems due to aggregation during folding in vivo.

3.5.1 OmpT-sPLA₂.

The ompT secretory signal was used in an attempt to produce sPLA₂ with a free N-terminus. After protein export, it was hoped that the translocated protein would be processed so that the secretory signal would be removed by the periplasmic signal peptidase, thus leaving Asn-1 sPLA₂. Unfortunately it was found that all the protein expressed from the pET-12a vector still possessed the secretory signal, as determined by SDS-PAGE. There are two possible explanations for the failure of the overexpressed protein to be properly processed. Firstly, protein export in E. coli requires that the protein to be translocated should be
Chapter 3: Overexpression, refolding and purification of sPLA₂

maintained in a soluble, loosely folded form so that it may be unfolded as it passes through the bacterial membrane via the secretory (Sec) protein complex. Proteins which are exported in *E. coli* can be loosely described as Sec-dependent or independent. Fig. 3.15 shows the proposed pathway of Sec-dependent secretion in *E. coli*.

**Fig. 3.15:** The proposed pathway for the Sec-dependent protein translocation in *E. coli*. (1) The cytoplasmic chaperone Sec B binds the protein when it emerges from the ribosome, and prevents folding. The Sec A/B complex takes the protein to the Sec YEG complex where the signal sequence is processed. (2) Hydrolysis of ATP causes the insertion of the protein into the translocation channel and the release of Sec A. The protein is then translocated by the protonmotive force (PMF). If the PMF is absent, Sec A completes protein translocation by multiple cycles of ATP hydrolysis (den Blaauwen and Driessen, 1996).

Sec-dependent secretion is based around the number of basic amino acids present in the protein to be translocated; Sec-dependent proteins contain ~10% basic residues and Sec-independent proteins contain less (von Heijne, 1994). The Sec-dependent proteins require ATP hydrolysis and the protonmotive force (Δp) to efficiently translocate proteins across the membrane. The Δp is generated across the inner membrane of bacteria by proteins embedded within the lipid bilayer (Fig. 3.16). Aerobic bacteria carry out the same processes of oxidative
phosphorylation that occur in eukaryotic mitochondria. Enzymes that catalyse the reactions of both glycolysis (Embden-Meyerhoff pathway) and the citric acid cycle are localised in the bacterial cytosol. Enzymes that oxidise NADH to NAD\(^+\) and transfer the electrons to the ultimate receptor O\(_2\) are localised to the bacterial plasma membrane. The movement of electrons through these membrane carriers is coupled to the pumping of protons out of the cell. Thus the net result is the generation of a pH gradient across the inner membrane. The \(\Delta \rho\) is composed of a membrane potential \((\Delta \Psi)\) and \(\Delta \rho \mathrm{H}\); \(\Delta \Psi\) being positive and \(\Delta \rho \mathrm{H}\) being acidic on the periplasmic side. \(\Delta \Psi\) is produced by a charge difference across the periplasmic membrane from ions such as H\(^+\), Na\(^+\) and Cl\(^-\). The permeability of biological membranes to ions is controlled by specific membrane-embedded transport systems. The \(\Delta \rho\) is utilised for several vital functions such as ATP generation via an membrane embedded ATPase, solute transport across the impermeable membrane, flagellum motion and protein translocation (Fig. 3.16).

![Diagram](image)

**Fig. 3.16:** Schematic representation of the generation and the uses of the protonmotive force in *E. coli.*
Proteins that contain a large number of acidic residues are able to be translocated solely by the $\Delta p$ after initially being inserted by the Sec system using ATP hydrolysis (Tokuda, 1994; Andersson and von Heijne, 1994a; 1994b; von Heijne, 1994). Sec-independent proteins spontaneously insert themselves into the lipid bilayer, although it could be possible that a small proportion of the molecules which do not insert spontaneously are picked up by the Sec-machinery (von Heijne, 1994). Thus ompTpbPLA$_2$ is able to be exported since its basic amino acid composition is $\sim$10% whereas ompTsPLA$_2$ contains $\sim$19% positive amino acids (the high basicity of sPLA$_2$ is reflected by its $pI$ value of 10.5 [Kramer and Pepinsky, 1991]). This high content of positively charged amino acids prevents export of sPLA$_2$ against the electrical component of the $\Delta p$ and in turn blocks the Sec-machinery. Therefore all the protein remains in the unprocessed form and forms inclusion bodies within the cell cytoplasm. Secondly, to be translocated, the protein must be in an export-compatible conformation probably in a loosely folded soluble state. This state has not yet been identified experimentally. This problem of avoiding premature folding is especially important in bacteria which exhibit no absolute link between protein synthesis and translocation. Sec B binds to the protein and maintains it in an export-compatible conformation (Jacq and Holland, 1993). PLA$_2$ forms inclusion bodies in E. coli since it cannot fold in the reducing environment of the cytoplasm. Since ompTpbPLA$_2$ can be translocated and ompTsPLA$_2$ cannot, this may imply that ompTsPLA$_2$ preferentially forms insoluble inclusion bodies instead of binding Sec B and thus fails to be exported into the periplasmic space. Therefore, a combination of low solubility and positive charge distribution may explain the inability to translocate sPLA$_2$.

3.6 Expression from eukaryotic cells.

The psPLA$_2$ construct (construct 2, Fig. 3.8) was made as it was thought that the expression of the inactive enzyme would increase the amount of protein produced by the MEL cell system. It was assumed that the secretion of the active form of the enzyme into the growth media resulted in cell lysis by hydrolysing the phospholipids present in the outer cell membrane. Construct 2 was made, but was not used, as it became apparent that expressing the
inactive psPLA$_2$ to increase the levels of protein produced may not work for two reasons. Firstly, if psPLA$_2$ was to be expressed in MEL cells, the protein would probably have difficulty in being translocated across the endoplasmic reticulum (ER) membrane or in folding within the organelle, as seen with the expression of psPLA$_2$ from yeast. The translocatory and folding systems in MEL cells and yeast are very similar and so the problems encountered in the expression of psPLA$_2$ in yeast would probably occur with other eukaryotic expression systems. Also, it is likely that the pro-sequence would cause further problems during protein folding as seen with the yeast expression system and with refolding of psPLA$_2$ overexpressed in E. coli. Secondly, it is unlikely that sPLA$_2$ is actually lysing the cells after secretion into the growth media. It has been shown that sPLA$_2$ will not hydrolyse lipids in the membranes of $^{14}$C labelled E. coli or yeast unless the membranes have been autoclaved (Harris et al., 1991). The inability to hydrolyse the phospholipids present in the membranes could be due to other membrane components such as proteins which interfere with sPLA$_2$ binding to the interface. Autoclaving removes these components and thus sPLA$_2$ now has access to the interface. Even after autoclaving, E. coli cells retain their original shape (Harris et al., 1991). sPLA$_2$ only seems to hydrolyse whole cell membranes after treatment with bactericidal/permeability-increasing protein isolated from polymorphonuclear leukocytes (Weiss et al., 1991; Wright et al., 1990). Thus it seems unlikely that sPLA$_2$ will be able to cause lysis of the MEL cells since their membranes also contain other components.

The translocatory machinery in mammals and other eukaryotes has been shown to be highly related to the export pathway in prokaryotes (Jungnickel et al., 1994). Thus the secretory pathway is probably very similar in MEL cells and methylotrophic yeast and the poor levels of enzyme produced may be for the same reasons and are listed below:

- poor solubility of the expressed protein which then preferentially forms inclusion bodies instead of entering the secretory pathway.

- poor efficiency of translocating a highly positively charged protein across the ER membrane.
Chapter 3: Overexpression, refolding and purification of sPLA$_2$

- Overexpression of recombinant protein, but no increase in the enzymes involved in protein folding or disulphide bond formation (i.e. protein disulphide isomerase).

- For pro-sPLA$_2$, the N-terminal extension may interfere with the folding of the protein, thus causing it to precipitate and form inclusion bodies within the ER.

The overexpression of recombinant proteins in yeast can be significantly improved by also overexpressing the proteins which are responsible for protein folding within the ER (Robinson et al., 1994). Thus it may be that, due to the high level of expression, sPLA$_2$ builds up in the ER and is unable to fold due to the low concentrations of foldases (e.g. protein disulphide isomerase, (PDI)). By increasing the expression of PDI sixteen-fold, a ten-fold increase in expression of platelet derived growth factor B homodimer (PDGF-BB) was observed in *Saccharomyces cerevisiae* (Robinson et al., 1994). PDGF-B is a small protein which forms a 30 kDa homodimer (PDGF-BB). The dimer is highly disulphide-bonded with a total of eight disulphides two of which are interchain bonds.

Since the secretory machinery in prokaryotes and eukaryotes is very similar, it may be asked why sPLA$_2$ is able to be translocated in eukaryotic cells (even if only in small amounts) and yet not at all from *E. coli*? This could be due to the membrane electrochemical potential (ΔΨ) present across the inner membrane of *E. coli* which is probably not present in the ER of eukaryotic cells (Andersson and von Heijne, 1994a; 1994b). Thus the membrane electrochemical potential being positive on the periplasmic face would be expected to oppose the translocation of positively charged proteins in *E. coli* but not in eukaryotes.
CHAPTER 4
OVEREXPRESSION, REFOLDING AND PURIFICATION OF BOVINE PANCREATIC PHOSPHOLIPASE A\(_2\) FROM \(E.\) coli.

RESULTS

4.1 Overexpression of recombinant \(^{15}\)N-pro-bovine pancreatic PLA\(_2\).

All experiments involving the expression of pro-bovine pancreatic PLA\(_2\) (pbPLA\(_2\)), were initially carried out in isotopically unenriched media. Once the expression and refolding protocol had been optimised, protein samples were made for nmr and kinetic studies. For nmr, the enzyme was isotopically labelled with the heavier nitrogen isotope \(^{15}\)N by growing the bacteria in the presence of \(^{15}\)NH\(_4\)Cl. Thus the following description of the expression and refolding of pbPLA\(_2\) is that of an \(^{15}\)N isotopically labelled sample.

pbPLA\(_2\) was expressed from a synthetic gene made by Dr. P. Mallinder. The synthetic gene incorporated codons found in highly expressed E. coli genes to ensure a high level of protein expression within the cell (Kane, 1995; Goldman et al., 1995). Initially pbPLA\(_2\) was expressed in the cytoplasm of E. coli under the control of the T7 promoter present on the pET 12a plasmid (Mallinder, unpublished work 1992). It was found that the protein did not accumulate within the cytoplasm of the cell upon induction and this was attributed to protease degradation of the protein within the cytoplasm. In order to prevent protease degradation, a secretory signal sequence was cloned upstream of the pbPLA\(_2\) gene, which would direct the expressed protein to the periplasmic space (Mallinder, unpublished work 1992). The 22 amino acid signal sequence was from the outer membrane protein T (ompT) and the pET 12a construct was expressed in the cell line BL21 (DE3) pLysS which lacks the ompT protease. As with human non-pancreatic PLA\(_2\), the unregulated expression of the protein was found to be toxic to the cells. Thus the tight control of protein expression was achieved by the T7 lysozyme encoded on the pLysS plasmid, and this prevented leaky expression from the pET 12a plasmid (Studier et al., 1990). After expression the secretory signal sequence would direct the protein to the periplasmic space. During translocation the protein would be processed to remove the ompT leader and the resultant pbPLA\(_2\) would then form insoluble inclusion bodies.
within the periplasmic space. It is unlikely that the pbPLA2 protein within the periplasmic space would refold correctly, as *E. coli* lacks the enzyme systems needed to fold a protein with a significant number of disulphide bonds.

The culture conditions and cell density at which protein expression was induced were found to be vital for efficient protein translocation. Initially cultures were grown in 250 mL of minimal media (A salts) at 30°C in a 2 L baffled flask. Once the cell density had reached O.D.600=1, protein expression was induced by the addition of 0.5 mM IPTG. Under these conditions only about 25% of the protein was translocated into the periplasmic space. It was found that late induction of the bacterial culture significantly increased the amount of protein translocated and processed (Fig. 4.1).

**Fig. 4.1**: 15% SDS-PAGE gel to show the relationship between protein translocation efficiency and time of induction. The cultures were grown in 250 mL of minimal media (A salts) at 30°C in 2 L baffled flasks. The O.D.600 at which the cultures were induced (with 0.5 mM IPTG) to express the protein are shown below the lanes. The cultures were then left for 12 hours to express the protein. In order to clearly visualise the protein bands, the inclusion bodies from the cultured cells were washed with Triton X-100 and then S-sulphonated prior to loading onto the gel.

It was found that late induction of the culture increased the amount of protein translocated to nearly 100%, whilst maintaining the same total level of protein expression. This
Chapter 4: Overexpression, refolding and purification of bPLA₂ from E. coli.

This is important since it has been demonstrated (data not shown) that in vitro refolding of OmpT-pbPLA₂ is much less efficient than that of processed pbPLA₂ and produces a protein which cannot be fully activated by trypsin. This increase in translocation efficiency was not seen when repeated with other types of growth media (e.g. 2 x YT). The late induction procedure increased the final amount of protein that could be isolated after refolding and purification from about 2-6 mg/L culture (with induction at low cell density) to approximately 15.7 mg (with induction at high cell density) for the ¹⁵N recombinant wild-type enzyme. Fig. 4.2 summarises the overall refolding and purification protocol used.

Fig. 4.2: Summary of the refolding and purification of recombinant bPLA₂.


4.2 Isolation and S-sulphonation of $^{15}$N-pbPLA$_2$.

Fig. 4.3 shows the isolation of $^{15}$N pbPLA$_2$ overexpressed in E. coli. After isolation of the inclusion bodies from the cells, the pellet was washed with the non-denaturing detergent Triton X-100. These detergent washes removed a large amount of contaminating protein (lanes 2 and 4). Unlike psPLA$_2$, pbPLA$_2$ was solubilised during S-sulphonation in 8 M urea (lane 7), thus providing a good purification step since many other E. coli proteins remain insoluble (lane 6). From lane 7, the S-sulphonated protein is about 80% pbPLA$_2$, as judged from the SDS-PAGE gel. After S-sulphonation the protein was isolated by dialysis against distilled water and 0.3% acetic acid.

Fig. 4.3: 15% SDS-PAGE gel showing the isolation of overexpressed $^{15}$N-labelled pbPLA$_2$ from E. coli. The lanes marked are: (1) Molecular weight markers, (2) Triton X-100 wash 1 supernatant, (3) Triton X-100 wash 1 pellet, (4) Triton X-100 wash 2 supernatant, (5) Triton X-100 wash 2 pellet, (6) insoluble protein after S-sulphonation, (7) soluble protein after S-sulphonation, (8) protein precipitate formed on pH reduction after refolding, (9) authentic bPLA$_2$, (10) Molecular weight markers.

Care was taken with the use of 8 M urea in the presence of the protein as the action of light, heat and alkaline pH can cause the breakdown of urea to form cyanate. Cyanate can chemically modify the amino acid side chains of the protein, which may affect refolding and
enzymatic activity. Therefore when preparing urea solutions, care was taken to avoid prolonged exposure to light and heat.

The reversible chemical modification of the protein thiols was carried out in two stages. In the first reaction, protein disulphide bonds that may have formed during isolation of the protein (by air oxidation), are cleaved by excess sulphite in the presence of denaturant (Fig. 4.4a).

![Fig. 4.4a: Chemical cleavage of disulphide bonds by excess sulphite (from Jaenicke and Rudolph, 1990).](image)

The free thiol produced is then reacted with 2-nitro-5-(thiosulpho)-benzoate (NTSB), which is converted to 2-nitro-5-thiobenzoic acid (NTB) (Fig. 4.4b). NTSB and NTB have different absorption maxima and so the reaction can be followed by measuring the concentration of NTB using its absorption at 412 nm.

![Fig. 4.4b: Chemical modification of the free thiol by NTSB (from Jaenicke and Rudolph, 1990).](image)
S-sulphonation was used to chemically modify the 14 cysteine thiols present within pbPLA$_2$ (Thannhauser et al., 1984; Thannhauser and Scheraga, 1985; Jaenicke and Rudolph, 1990) and this was carried out for several reasons:

- It allowed the chemical cleavage of disulphide bonds that may have formed by air-oxidation during isolation of the protein.

- S-sulphonation also introduces a negative charge for every thiol, thus increasing the solubility of the protein compared to reduction by β-mercaptoethanol.

- Lyophilised S-sulphonated protein can also be readily stored for long periods of time at -20°C, compared to reduced proteins which are preferably refolded immediately, as S-sulphonation prevents disulphide bonds forming in the absence of the correct conditions.

- S-sulphonation is a reversible modification and so disulphide bond formation can be initiated in the presence of the appropriate "oxido-shuffling" system, e.g. oxidised and reduced glutathione. The mechanism of disulphide bond formation using an oxido-shuffling system (in a reduced polypeptide) is shown in Fig. 4.5.

**Fig. 4.5:** Schematic representation of disulphide bond formation using reduced and oxidised low molecular weight thiols (RS$^-$ and RSSR). The polypeptide may be S-sulphonated, in which case the thiols (S') would be modified to SSO$_3^-$ (from Jaenicke and Rudolph, 1990).
Chapter 4: Overexpression, refolding and purification of bPLA₂ from E. coli.

The formation of certain non-native disulphides could trap the protein in a non-native conformation. However disulphide bond reduction (the rate marked * in Fig. 4.5) should be slower for native disulphides. Therefore reduction of the disulphides must be allowed to take place in order to allow for rearrangement and re-oxidation until the protein eventually assumes its native conformation.

4.3 In vitro refolding of ¹⁵N-bPLA₂.

Due to the large number of cysteine residues present within pbPLA₂ (14 in total), the protein can adopt a large number of disulphide bridge patterns during refolding. With 7 disulphide bonds there is a theoretical possibility of forming 135,135 different bridging patterns (Jaenicke and Rudolph, 1989). In reality, the number of bonds possible is much lower because some combinations of disulphide bonds would be physically impossible. So, apart from a method of refolding the protein into its native conformation, it is vital to devise a purification protocol for bPLA₂ that can be used to separate and purify the native form of the enzyme from other stable but incorrectly folded conformations.

The amount of S-sulphonated protein used for the refolding experiments was such that the protein concentration was between 30-40 mg/L after dilution. It was found that the greatest amount of active enzyme could be produced using the following conditions:- 25 mM sodium tetraborate pH 8.7, 5 mM potassium EDTA, 2 mM oxidised glutathione and 4 mM reduced glutathione. All the buffers were degassed and the protein refolded by rapid dilution from 8 M to 1 M urea under argon, after which the solution was left in darkness at room temperature for a further 18 hours. For a 4 L maximum reaction volume, approximately 120-160 mg of S-sulphonated protein could be refolded each time.

Table 4.1 shows the effects of refolding the S-sulphonated protein in the presence or absence of molecular oxygen. Also, protein isolated from rich (2 x YT) and minimal media (A salts) cultures were refolded to show the effect of growth media on over-all protein refolding levels.
Chapter 4: Overexpression, refolding and purification of bPLA₂ from E. coli.

Table 4.1: Levels of refolded protein isolated under defined expression and folding conditions. The cultures (250 mL in a 2 L flask) were grown at 30°C and protein expression induced at O.D₆₀₀ ~2.8 with 0.5 mM IPTG. The S-sulphonated protein was refolded by rapid dilution, from 8 M to 1 M urea. †The final protein concentration after refolding was 30 mg/L. After activation the protein was not further purified and so the actual level of isolated enzyme after purification would be lower (~60% of the figure quoted).

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<th>A salts expressed protein.</th>
<th>A salts expressed protein.</th>
<th>2 x YT expressed protein.</th>
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<td></td>
<td>Refolding -O₂</td>
<td>Refolding +O₂</td>
<td>Refolding -O₂</td>
</tr>
<tr>
<td>Amount of S-sulphonated protein refolded</td>
<td>45 mg†</td>
<td>45 mg†</td>
<td>45 mg†</td>
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<tr>
<td>Protein eluted from Fast Flow S-Sepharose column</td>
<td>28.9 mg</td>
<td>18.7 mg</td>
<td>12.9 mg</td>
</tr>
<tr>
<td>Soluble protein after dialysis/lyophilisation</td>
<td>18.9 mg</td>
<td>10.1 mg</td>
<td>6.1 mg</td>
</tr>
<tr>
<td>% of protein which can be activated by trypsin</td>
<td>40.4 %</td>
<td>39 %</td>
<td>29.4 %</td>
</tr>
<tr>
<td>Total protein activated</td>
<td>7.6 mg</td>
<td>4 mg</td>
<td>1.8 mg</td>
</tr>
<tr>
<td>Amount of refolded protein expected per L of culture</td>
<td>18.8 mg</td>
<td>9.7 mg</td>
<td>5.7 mg</td>
</tr>
</tbody>
</table>

From Table 4.1 it can be seen that the final levels of protein isolated depends on the culture media used and the presence or absence of molecular oxygen during refolding. The presence of oxygen leads to a significant increase in the amount of protein lost during refolding relative to that when oxygen is absent (58% compared to 36%). Thus it may be that in the presence of molecular oxygen, incorrect disulphide bridge formation is increased causing the formation of protein species which favour aggregation and so precipitate out of solution. The percentage of the minimal media expressed protein that can be trypsin activated is the same irrespective of the refolding conditions. But the protein isolated from rich media cultures shows a reduction in the amount of soluble protein left after refolding (71% loss of protein) and also the percentage of the protein which can be activated by trypsin. This is probably due to the poor translocation efficiency of the protein during expression on 2 x YT
media, thus producing more of the ompT-pbPLA2 which can be refolded but not activated (data not shown).

The actual percentage of protein that refolded correctly under anaerobic conditions can only be estimated, as the S-sulphonated protein did not contain 100% pbPLA2. From the SDS-PAGE gel (Fig. 4.3, lane 7), the S-sulphonated protein contained approximately 70-80% pbPLA2. Therefore of the 45 mg of protein (from cells grown on minimal media) refolded in the absence of oxygen, 32-36 mg was pbPLA2. From the first column step (Fast Flow S-Sepharose), ~29 mg of protein was isolated and consists solely of PLA2, as the E. coli proteins were removed by the previous reduction in pH. A further 10 mg was lost by aggregation during dialysis, thus leaving ~19 mg of protein of which 40% could be activated (i.e. 7.6 mg). So from the initial ~34 mg of S-sulphonated pbPLA2 refolded, 7.6 mg of enzyme could be trypsin activated. 22% of the initial PLA2 was correctly refolded, although a further 40% of this would be expected to be lost during the final purification steps. Under the alternative refolding conditions, the percentage of S-sulphonated protein that could be refolded was reduced even further. With minimal media expressed protein refolded in the presence of oxygen, only ~12% of the initial pbPLA2 was refolded.

4.4 Isolation and purification of 15N-pbPLA2.

After refolding pbPLA2 by rapid dilution, it was necessary to isolate the diluted protein from such a large volume of buffer (~4 L). This was achieved by using a Fast Flow S-Sepharose ion exchange column. The advantage of using Fast Flow S-Sepharose resin was that high flow rates could be achieved and also the resin has a high protein binding capacity. The isoelectric point of bPLA2 is 8.5, so at pH 4.5 the protein will have a net positive charge and thus will bind to the negatively charged resin (-SO3- being the functional group attached to the gel matrix). With the reduction in pH, the refolding solution became very cloudy and so the mixture was centrifuged to remove the precipitation. It was found that the precipitate consisted mainly of E. coli proteins and a small amount of pbPLA2 (see Fig. 4.3 lane 8). Therefore the reduction in pH proved to be a simple and efficient method of removing all the
contaminating E. coli proteins that had also been isolated and refolded from the inclusion bodies.

The refolded protein solution was diluted by a factor of two with distilled water to reduce the ionic concentration so that the protein would bind to the column resin at pH 4.5, and was eluted with 200 mL of 1 M sodium chloride. A linear salt gradient would have eluted the protein in a large volume and there would also be no significant separation due to the poor resolution of soft gel columns. The eluted protein (~100 mg) was dialysed to remove the salt and finally lyophilised prior to activation.

4.4.1 Activation and purification of $^{15}$N-phPL$$_A$$._2$

Once refolded, the enzyme with the native fold must be isolated from the other protein conformers. Also a method for assessing the purity of the enzyme must be found in order to judge when the protein had been purified to homogeneity. An enzyme assay would accurately show whether the enzyme had been purified to homogeneity as the measured specific activity of the refolded protein should be similar to the native enzyme. The presence of incorrectly folded protein (which was assumed to be catalytically inactive) would give a lower specific activity for the measured enzyme concentration. The egg-lecithin assay cannot be used to determine if the enzyme has been purified as the accuracy (~10-15%) and sensitivity (~2-3 μg/assay) are insufficient to determine if the sample contains only one protein species towards the final stage of the purification procedure.

A Mono P 5/20 chromatofocusing column was used to determine if other folded protein forms were present within the sample (Section 4.4.5). It was assumed that the incorrectly folded protein species (that were resistant to the action of trypsin) would have a slightly different surface charge compared to the native enzyme due to differences in the tertiary structure. Thus the isoelectric point (pI) of the various protein species would be slightly different, and the chromatofocusing column (which separates proteins by their pI) could be used to identify the fractions from the ion exchange column that contained the correctly refolded enzyme. The chromatofocusing column could also be used to identify protein that had been chemically modified by the refolding process, for example protein that
had undergone deamidination (-\text{CONH}_2 \Rightarrow -\text{COO}^-). This would introduce another negative charge into the protein and reduce the pI of the molecule and would change the elution volume from the chromatofocusing column.

4.4.2 Trypsin activation of $^{15}$N-pbPLA$_2$.

The refolded protein was expressed as the pro-enzyme, so the seven amino-acid N-terminal pro-sequence had to be removed to form the active enzyme. This was achieved by treating the pro-enzyme with trypsin, the natural activator of the pancreatic enzyme (de Haas, \textit{et al.}, 1968). Trypsin had the added advantage that, due to its less specific nature, incorrectly folded protein would be degraded. With a large number of Lys and Arg residues present at the surface of PLA$_2$, trypsin should cleave the loosely folded non-native conformers.

Activation of the pbPLA$_2$ by trypsin was followed with the egg-lecithin assay using the pH-stat. Once there was no further increase in PLA$_2$ activity, the activation was halted by the addition of 2 M ammonium sulphate, 40 mM sodium acetate pH 4.5.

4.4.3 Purification of activated $^{15}$N-bPLA$_2$ by hydrophobic interaction chromatography.

The separation of the active form of the enzyme was achieved using a hydrophobic interaction column (High Load Phenyl Sepharose). Fig. 4.6a shows the elution profiles of recombinant $^{15}$N-pbPLA$_2$ activated with trypsin from a High Load Phenyl Sepharose XK16 column. The activated protein was purified using a larger capacity column (High Load Phenyl Sepharose XK 26/10), the elution profile of which is shown in Fig. 4.6b.
Fig. 4.6: (a) Trypsin activation of recombinant $^{15}$N-pbPLA$_2$. The elution profiles are from two separate runs, each consisting of ~100 µg of protein before and after treatment with trypsin (from the High Load Phenyl Sepharose XK 26/10 column). The buffers used were: buffer A: 20 mM sodium acetate pH 4.5, 15% isopropanol (v/v) and buffer B: 1 M ammonium sulphate, 20 mM sodium acetate pH 4.5. (b) Preparative purification of $^{15}$N-bPLA$_2$ after trypsin activation. ~160 mg of protein was applied to the High Load Phenyl Sepharose XK 26/10. The buffer conditions were as described in (a) and the fraction volumes were 12 mL. For both figures, the gradient has been shifted to account for the column bed volume.
Chapter 4: Overexpression, refolding and purification of bPLA$_2$ from E. coli.

It can be seen from Fig. 4.6a, that there is a change in the elution volume between the two forms of the enzyme, thus hydrophobic interaction chromatography allows the separation of the pro-enzyme from the trypsin activated protein. Fig. 4.6b shows the preparative activation of $^{15}$N-bPLA$_2$. The fractions containing PLA$_2$ activity (as judged by the egg-lecithin assay), were pooled and further purified using cationic exchange chromatography. The elution point of the preparative activated protein (Fig. 4.6b) was found to be significantly different to that of the analytical sample (35% B compared with 5% B) (Fig. 4.6a). This may be due to the large amount of protein applied to the column during the preparative purification, thus changing the separation characteristics of the column.

4.4.4 Cation exchange chromatography purification of $^{15}$N-bPLA$_2$.

The fractions eluted from High Load Phenyl Sepharose XK 26/10 column which had PLA$_2$ activity were pooled and dialysed prior to further purification using ion exchange chromatography. Cation exchange chromatography allowed the separation of the enzyme with the native conformation from any incorrectly folded protein which was resistant to the action of trypsin. Fig. 4.7 shows the elution profile of recombinant $^{15}$N-bPLA$_2$ from the High Load S-Sepharose 26/10 column. From Fig. 4.7 other protein species can be seen to elute throughout the salt gradient, even though the protein applied contained only bPLA$_2$ as judged by SDS-PAGE (gel not shown). bPLA$_2$ activity was located within the major peak, but from previous refolding experiments it was evident that the enzyme had still not been purified to homogeneity (see Section 4.5).
Chapter 4: Overexpression, refolding and purification of bPLA$_2$ from E. coli.

Fig. 4.7: Elution profile of recombinant $^{15}$N-bPLA$_2$ from the High Load S-Sepharose 26/10 column. Approximately 65 mg of protein was applied to the column in 20 mM sodium acetate pH 4.5, after which the protein was eluted with a linear salt gradient of 0-1 M sodium chloride in the same buffer. The fraction volumes collected were 10 mL. The gradient has been shifted to account for the column bed volume. The major peak contained bPLA$_2$ activity as judged by the egg-yolk lecithin assay.

4.4.5 Chromatofocusing of $^{15}$N-bPLA$_2$.

Fractions from the major peak from the High Load S-Sepharose column were analysed to identify those which contained only the native folded form of recombinant bPLA$_2$. About 50 µg of protein from each fraction was applied separately to a Mono P 5/20 chromatofocusing column which separated proteins by their pI. Instead of using a salt gradient, a pH gradient was used to separate the proteins by the differences in their pI from the weak anion exchange resin. The pH gradient was generated by using an ampholyte buffer (Polybuffer). This gave the column the ability to resolve very small differences between proteins due to their surface charge. Fig. 4.8 shows the elution profiles from the Mono P
column of the fractions isolated from the High Load S-Sepharose column which contained PLA₂ activity.

![Graph showing chromatofocusing profiles](image)

**Fig. 4.8**: Chromatofocusing profiles of fractions (a-e) containing PLA₂ activity isolated from the High Load S-Sepharose column (Fig. 4.7). Approximately 50 μg of protein was applied per run, after which 30 mL of Polybuffer 96 pH 6.0 was passed through the column to create the pH gradient from 9.6 to 6.0.

It can be seen that the Polybuffer alone had an absorbance at 280 nm (Fig. 4.8a). It was clear that another protein species was present within some of the fractions examined (Fig. 4.8c and d). Fractions which contained only one peak which corresponded to the elution volume of active bPLA₂ (e.g. Fig. 4.8e) were then pooled, the remaining fractions were discarded. Using chromatofocusing it was shown that the leading edge of the peak from the High Load S-Sepharose column (Fig. 4.7) was contaminated with another protein species which was resistant to the action of trypsin and had a slightly different surface charge (i.e. a lower pI).
Therefore the enzyme fractions pooled contained only one protein species as judged by chromatofocusing and by titration with the phospholipid analogue RLM-1 (see Section 4.5).

4.5 Nmr of recombinant bPLA₂ with RLM-1

The initial purification strategy was found to be inadequate to purify the protein to homogeneity as nmr experiments involving 15N recombinant bPLA₂ and the phospholipid analogue RLM-1 showed. RLM-1 (Fig. 4.9) is a non-hydrolysable phospholipid analogue which binds very tightly as a monomer to the active site of the enzyme (Kₐ < 1 μM, unpublished data). The substitution of the ester for an amide group at the sn-2 position prevents the cleavage of the bond by PLA₂. RLM-1 can thus be used as an active site titrant of PLA₂ and its binding can be followed by its perturbation of the 1H and 15N nmr spectra of the protein.

![Fig. 4.9: Structure of the phospholipid analogue RLM-1 (R. Magolda, DuPont Pharmaceuticals). The amide group prevents the hydrolysis of the bond by PLA₂.](image)

The initial purification method involved trypsin activation of the recombinant enzyme followed by hydrophobic interaction chromatography (Kogelberg et al., unpublished work). This protocol was used to purify 15N labelled recombinant bPLA₂, which was subsequently used in 2D-HSQC nmr experiments. The nmr experiment involved the titration of RLM-1 to
the enzyme sample (Primrose et al., unpublished work). The binding of RLM-1 to the active site of the enzyme was followed by nmr to monitor the changes in the proton resonances of amino acid residues at the ligand binding site. Since the ligand has a high affinity for the active site of the enzyme, the addition of RLM-1 to the nmr sample would favour the formation of the enzyme/ligand complex at a 1:1 ratio of protein to ligand. Upon saturation of PLA2 with RLM-1, only proton signals from ligand in the ligand/enzyme complex should be present. With the 2D-HSQC nmr experiment using 15N recombinant bPLA2 (purified by the method of Kogelberg et al.), it was found that the protein could not be saturated with excess RLM-1 (Primrose et al., unpublished work, data not shown). Only ~ 75% of the recombinant bPLA2 in the sample was able to bind RLM-1; the remaining 25% of the enzyme remained unliganded free in solution. It was concluded that the 25% of the enzyme which was unable to bind RLM-1 had been chemically modified by the refolding process or the protein was incorrectly folded in some undetermined way. The contaminating protein (which will be referred to as modified PLA2 or mPLA2) may not be a single species. Thus a method had to be devised to remove mPLA2 from the refolded protein for several reasons. For studies of structure and function, a high purity of enzyme was required. The presence of mPLA2 would have led to the incorrect determination of the active protein concentration as there was no method of determining the exact amount of mPLA2 produced from each refolding. This would have affected the measured kinetic parameters such as $k_{cat}$ for the refolded mutant proteins, thus introducing further errors. As has been previously shown by Primrose et al., the proton signals from mPLA2 free in solution complicate the nmr spectra of the enzyme/RLM-1 complex. So if further work was to be done on determining the enzyme's solution structure complexed with an active site ligand, the protein would require further purification.

The concentration of mPLA2 could not be easily determined. A pH-stat assay could be used for example, with 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol as the substrate. Thus by comparing the specific activity of authentic bPLA2 with that of the recombinant wild-type enzyme, the purity of the protein could be assessed. But the inherent inaccuracy of the pH-stat assay method (between 10-15% error), would be insufficient to determine the exact protein purity. The simplest method of detecting the mPLA2 form was to use nmr and RLM-1,

122
although concentrations up to 1 mM of protein are required to obtain a good signal. One-dimensional proton nmr (1-D $^1$H nmr) allowed the detection of both the correctly refolded form of the enzyme and mPLA$_2$ by following the saturation of the protein by RLM-1. Therefore it could be established whether the refolded recombinant wild-type bPLA$_2$ required further purification to remove mPLA$_2$ and the level of contamination estimated. This was achieved by comparing the 1D titration spectra of purified recombinant bPLA$_2$ and authentic bPLA$_2$ in the presence of RLM-1.

It was clear that the activated recombinant protein from the Phenyl Sepharose column still contained other refolded PLA$_2$ species as they could be separated using the ion exchange column (Fig. 4.7). Even after cation-exchange chromatography the major protein peak (which contained the PLA$_2$ enzymatic activity), was still contaminated with at least one other species. Fractions containing this contaminating protein could only be identified using a chromatofocusing column (Mono P column) and relied on the difference in the charge distribution on the surface of the molecules (Fig. 4.8). Therefore fractions from the High Load S-Sepharose were assessed for purity using the Mono P column, prior to pooling the protein fractions. Once pooled, 1-D $^1$H nmr titrations of the recombinant wild-type enzyme with RLM-1 were carried out to see if the new column steps had purified the protein to homogeneity. This was achieved by comparing the 1-D $^1$H nmr spectra of authentic bPLA$_2$ and RLM-1 with that of the recombinant enzyme and the ligand. Fig. 4.10a and b show the 1-D nmr titrations of authentic and recombinant bPLA$_2$ respectively. Changes in the positions of the resonances of the C$_5$ protons of Ile-9 and Leu-41 were used to confirm the formation of the enzyme-RLM-1 complex. The formation of protein-inhibitor complexes have been previously studied, using the changes in the positions of these (and other) resonances to determine when binding had occurred (Slaich et al., 1992; Bennion et al., 1992).

For the enzyme free in solution, the proton resonances of Ile-9 and Leu-41 were at 0.02 ppm and 0.06 ppm respectively. In the enzyme-RLM-1 complex the proton resonances of Ile-9 and Leu-41 shift to -0.06 ppm and 0.45 ppm respectively. The exact concentration of the RLM-1 was not known, so saturation of the enzyme was judged to have occurred once there was no further change in the spectrum of the protein. Fig. 4.10a shows the addition of RLM-1 to authentic bPLA$_2$. It can be clearly seen that after the first addition of RLM-1 there are
Chapter 4: Overexpression, refolding and purification of bPLA2 from E. coli.

Proton resonances from both free and complexed enzyme. By the second addition all the free protein had been saturated by the inhibitor and no proton resonances from free protein were observed. Fig. 4.10b shows the addition of RLM-1 to the recombinant bPLA2 isolated and purified by hydrophobic interaction chromatography followed by cation exchange chromatography and fractions assessed for contamination by the Mono P column. Again, as RLM-1 was added, the proton resonances from Ile-9 and Leu-41 moved and no free enzyme was observed once the protein had been saturated with the ligand. Therefore this confirmed that the introduction of two more chromatographic steps was absolutely necessary in order to produce a homogeneous protein sample.
Fig. 4.10a: 1-D $^1$H spectra of the addition of RLM-1 to authentic bPLA$_2$ (0.5 mM). Conditions: 20 mM d$_4$ succinate "pH 6.1", 50 mM calcium chloride and 125 mM sodium chloride at 314K.
Fig. 4.10b: 1-D $^1$H spectra of the addition of RLM-1 to recombinant bPLA$_2$ (0.5 mM). Conditions: 20 mM $d_4$ succinate "pH 6.1", 50 mM calcium chloride and 125 mM sodium chloride at 314K.
Chapter 4: Overexpression, refolding and purification of bPLA₂ from E. coli.

4.6 Overall purification table of bPLA₂.

The overall purification of ¹⁵N-bPLA₂ is shown in Table 4.2. The amount of enzyme isolated was lower than that of some of the mutants produced, for example 15.7 mg/L for ¹⁵N bPLA₂ compared to 22.1 mg/L for N71E bPLA₂. The reason for this was probably because the bacterial culture had not reached a sufficiently high cell density before the induction of protein expression. Therefore not all the protein was efficiently translocated into the periplasmic space leaving a small amount of the unprocessed ompT form. The S-sulphonated protein was refolded in 4 x 4 L volumes, after which all the samples were pooled and purified together.

<table>
<thead>
<tr>
<th></th>
<th>Amount of protein (mg)</th>
<th>Specific activity (µmoles/min/mg)</th>
<th>Total activity (µmoles/min)</th>
<th>Purification</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-sulphonated protein to be refolded</td>
<td>707</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Soluble protein eluted from the Fast Flow S-Sepharose column, prior to trypsin activation</td>
<td>166</td>
<td>63</td>
<td>10458</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Protein eluted from the Phenyl Sepharose XK 26/10</td>
<td>67.5</td>
<td>115</td>
<td>7763</td>
<td>1.8</td>
<td>74</td>
</tr>
<tr>
<td>Pooled fractions shown to be pure bPLA₂ using the Mono P 5/20 column</td>
<td>43.2</td>
<td>146</td>
<td>6307</td>
<td>2.3</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 4.2: Purification table for ¹⁵N-bPLA₂ expressed in E. coli and refolded in vitro in the absence of molecular oxygen. The values are from 2.75 L of bacterial culture, induced to express protein at O.D.₆₀₀=1.9. ~700 mg of S-sulphonated protein was refolded in 4 x 4 L volumes, at a final protein concentration of ~45 mg/L. The specific activity measurements were made using the egg-yolk lecithin assay. Thus the final amount of bPLA₂ was 15.7 mg/L of bacterial culture. Purification and yield were calculated as follows:

Purification = Specific activity of protein at stage of isolation
Specific activity at the start of the purification

Yield (%) = Total activity of protein at stage of isolation x 100
Total activity at the start of the purification
4.7 Overexpression of recombinant proteins in *E. coli*.

In order to fully characterise an enzyme structurally and functionally, a large quantity of the protein of interest is required. Isolation of the protein from source can be difficult and time consuming due to the low levels of protein usually expressed within the source material. The development of molecular biology techniques has allowed genes from any cell type to be transferred into bacteria and under the control of the appropriate promoter, large quantities of the foreign protein can be overexpressed. The organism of choice for cloning and expression work is still *E. coli*, as its molecular genetics are well understood and the cells are easy to handle and grow. *E. coli* does have its limitations as some eukaryotic proteins are unable to fold correctly within the cell cytoplasm and so form insoluble inclusion bodies (Marston, 1986; Schein, 1989; Mitrald and King, 1989).

In some cases, the localisation of the recombinant protein into inclusion bodies may be beneficial. Some foreign proteins when expressed in the cytoplasm of *E. coli* are rapidly degraded, for example bacteriorhodopsin (Chen and Gouaux, 1996). But when expressed as a fusion with maltose-binding protein, the polypeptide aggregates to form inclusion bodies which are resistant to the action of cellular proteases (Chen and Gouaux, 1996; Schein, 1989; Enfors, 1992). Eukaryotic proteins with disulphide bonds that are expressed within *E. coli* always form inclusion bodies, as the environment of the cytoplasm is reducing and so the polypeptide cannot fold into its native structure (Bardwell, 1994). Cytoplasmic proteins containing disulphide bonds are rare, and are typically sulphhydryl oxidoreductases, which undergo redox interconversion between free sulphhydryl and disulphide-bonded conformations (e.g. thioredoxin). There are good reasons why disulphide bonds are not normally permitted in cytoplasmic proteins: the thiol group is one of the most reactive side-chains and is used at the active site of a number of enzymes. Oxidation of these or other thiols in a protein can disrupt function (Bardwell, 1994). Therefore there are several mechanisms within the cell cytoplasm that actively prevent the formation of disulphide bonds in the cytoplasm, for example glutathione and thioredoxin whose reduced states are maintained by their respective NADPH-
dependent reductases, glutathione reductase and thioredoxin reductase (Gilbert, 1990; Hwang et al., 1992; Derman et al., 1993; Bardwell, 1994). *E. coli* lacks the accessory proteins and the internal compartmentalisation (i.e. endoplasmic reticulum, ER) for folding of disulphide bond containing proteins. The periplasmic space (which is the homologous structure to the ER in *E. coli*) is where disulphide bond formation occurs and makes up to 30% of the cell volume (Bardwell, 1994). Here dedicated enzyme systems (disulphide bond forming proteins, or Dsb proteins) are present which rapidly oxidise the thiols to form disulphide bonds within the secreted peptide. Although the periplasmic space is the location of disulphide bond formation, *bPLA₂* forms insoluble inclusion bodies due to the large number of disulphide bonds found in the protein. It has been shown that a few disulphide bridged proteins translocated into the periplasmic space do fold correctly, giving the native disulphide bonding pattern and full functionality. These include bovine pancreatic trypsin inhibitor which has 3 disulphide bonds (Ostermeier and Georgiou, 1994), T-cell receptor fragments with 2-4 disulphide bonds (Wülfing and Plückthun, 1994) and a novel PLA₂ type toxin from *N. s. scutatus* snake venom with 7 disulphide bonds (Hodgson et al., 1993). Some of the expressed proteins are only correctly folded when expressed as fusion proteins, such as the PLA₂ type toxin from *N. s. scutatus* snake venom which was fused to two IgG binding domains (Hodgson et al., 1993), or by overexpression of other proteins such as chaperones and DsbA as with the T cell receptor (Wülfing and Plückthun, 1994). Therefore, it is not surprising that *bPLA₂* expressed on its own forms insoluble inclusion bodies after translocation, as *E. coli* does not possess the appropriate enzyme systems to deal with such a highly disulphide-bridged protein.

*bPLA₂* was expressed with a secretory signal sequence preceding the protein, thus directing the translated polypeptide to the periplasmic space. There are a number of good reasons why an expressed recombinant protein may want to be sequestered into the periplasmic space of *E. coli*. Firstly, if the protein expressed is sensitive to proteolysis, then the periplasmic space is an ideal location to target protein expression as there are fewer proteases present within the periplasmic space compared to the cytoplasm (Goldberg and Goff, 1986; Marston, 1989). If the recombinant protein is highly toxic to the cell, then secretion of the polypeptide into the periplasmic space where it cannot interfere with cellular functions is ideal. Since disulphide bond formation occurs within the periplasmic space, it is
possible to overexpress proteins containing a few disulphide bridges where they will then fold with the native bonding pattern (for example the T cell receptor), if the conditions favour bond formation. If the signal sequence is correctly processed, the N-terminus of the recombinant protein will be identical to the native product. Finally, isolation of the recombinant protein can be simplified if directed to the periplasmic space. By using gentle osmotic shock and lysozyme to degrade the polysaccharide cell wall, spheroplasts are formed from which the periplasmic space proteins will be released and the cytoplasm left intact.

The main problem encountered of expressing the ompT-pbPLA2 in E. coli was the poor translocation efficiency. As discussed in Chapter 3, bPLA2 might be expected to be translocated efficiently via the secretory pathway as the polypeptide is not as positively charged as sPLA2 (sPLA2 ∼20% positively charged residues [pI of 10.5] compared to ∼10% for bPLA2 [pI of 8.5]). From the SDS-PAGE gel (Fig. 4.1), at low cell density translocation of the ompT-pbPLA2 polypeptide is relatively poor, but a significant increase is observed when the cells have reached the stationary phase of growth. This increase in translocation efficiency may be due to a "stress" related response within the bacterial cells.

Although the primary sequence of a protein contains all the information to correctly fold the polypeptide into its native conformation, protein folding in vivo has been shown to require other protein factors for the efficient folding of the polypeptide. These protein factors are referred to as molecular chaperones, and are defined as "a family of unrelated classes of protein that mediate the correct folding and assembly of other polypeptides, but are not themselves components of the final functional structure" (Ellis and van der Vies, 1991). The bulk of the molecular chaperones also belong to the set of heat-shock proteins (hsp), so-called because of their elevated expression under conditions of cellular stress, which increase the cells chance of survival. Some hsp (for example GroEL and GroES) are expressed constitutively, as they perform functions which are essential to cell growth. The hsp are found in both eukaryotes and prokaryotes and belong to several conserved families. Table 4.3 shows a list of some of the hsp families and some of their associated functions.
Chapter 4: Overexpression, refolding and purification of bPLA$_2$ from E. coli

Table 4.3: A summary of some of the proteins for which there is good evidence of molecular chaperone function in vivo. Abbreviations used: hsc, constitutively-synthesised protein with high homology to an hsp (modified from Lund, 1995).

<table>
<thead>
<tr>
<th>Molecular Chaperone</th>
<th>Proposed functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp70 family</td>
<td></td>
</tr>
<tr>
<td>DnaK (bacteria)</td>
<td>• Essential for growth at all temperatures.</td>
</tr>
<tr>
<td></td>
<td>• Required for the induction of the heat-shock response.</td>
</tr>
<tr>
<td></td>
<td>• Role in secretion of some periplasmic proteins.</td>
</tr>
<tr>
<td></td>
<td>• Acts with DnaJ/GrpE</td>
</tr>
<tr>
<td>Cytosolic hsp70's: SSA1-SSA4; SSB1 and SSB2 (yeast); hsp70 and hsc70 (animal cells).</td>
<td>• Import of proteins into mitochondria, chloroplasts and ER.</td>
</tr>
<tr>
<td></td>
<td>• Disassembly of clathrin cages.</td>
</tr>
<tr>
<td></td>
<td>• Binding of nascent protein chains.</td>
</tr>
<tr>
<td></td>
<td>• Protection against and recovery from heat-shock</td>
</tr>
<tr>
<td>DnaJ-type proteins</td>
<td>• Interact with hsp70 to prevent aggregation of unfolded or nascent proteins.</td>
</tr>
<tr>
<td></td>
<td>• May have a role in secretion and in reactivation of heat-inactivated proteins.</td>
</tr>
<tr>
<td>hsp60 family</td>
<td>• Binding of unfolded or partially folded proteins; prevention of protein aggregation.</td>
</tr>
<tr>
<td>GroEL-like proteins (GroEL in bacteria, hsp60 in mitochondria and chloroplasts)</td>
<td>• Act with co-chaperones: GroES in bacteria; homologous proteins found in mitochondria and chloroplasts.</td>
</tr>
<tr>
<td>SecB</td>
<td>• Required for secretion of a subset of bacterial proteins: binds them in an unfolded form and delivers them to membrane translocation machinery.</td>
</tr>
</tbody>
</table>

It has now been shown that a variety of environmental stimuli can produce a similar effect to the heat-shock response and these include carbon starvation (Nystrom, 1995; Matin, 1991; Jenkins et al., 1991), chemical inducers such as ethanol and heavy metals (van Dyk et al., 1995).

So how can the heat-shock response improve protein translocation? As discussed previously (Chapter 3, Section 3.5.1), during or after translation, the polypeptide chain is maintained in a soluble, loosely folded form by the cytoplasmic molecular chaperone SecB (see Fig. 3.15, Chapter 3 Section 3.5.1), which prevents folding or aggregation due to hydrophobic interactions (den Blaauwen and Driessen, 1996).
Chapter 4: Overexpression, refolding and purification of bPLA2 from E. coli.

The signal sequence of the preprotein/SecB complex binds to SecA. The tertiary complex traverses to, or along, the membrane until it encounters the integral membrane translocase subunits (Sec YEG), after which the protein is translocated into the periplasmic space. SecB comprises only 0.08% of the total cellular cytosolic proteins (Collier, 1993), and the expression level of this chaperone is not increased upon heat-shock (Altman et al., 1991). Therefore when recombinant protein expression is induced with IPTG (at O.D.600=1), the cytoplasmic pool of SecB could become depleted very rapidly and so the majority of the recombinant protein aggregates to form insoluble inclusion bodies within the cell cytoplasm in the ompT-pbPLA2 form (Fig. 4.11 A). As the bacterial culture reaches stationary phase (at O.D.600≈2.8), a stress response is initiated. This may be from a lack of a carbon source (i.e. glucose), but whatever the initial cause of the response, hsp production is initiated (Fig. 4.11B).

Hsp expression is regulated by the RNA polymerase transcription factor sigma-32 (σ32), which has a molecular weight of 32 kDa (Manger and de Kruijff, 1995). Specific E. coli DNA promoters can be recognised by the RNA polymerase/sigma factor (or holoenzyme), thus regulating gene expression. Thus the major sigma factor, σ70, is responsible for transcription of most genes during exponential growth. There are several sigma factors in E. coli, each of which directs transcription of genes whose products are needed for specific functions (Helmann and Chamberlin, 1988). Thus the RNA polymerase/σ70 complex is unable to transcribe some of the hsp genes, as the holoenzyme does not "recognise" the promoter. Those hsp genes which are transcribed constitutively (e.g. GroEL and GroES which are required for normal cell function) and are also induced by cellular stress, have two promoters, one recognised by the σ70 holoenzyme and the other by the σ32 holoenzyme (Manger, and de Kruijff, 1995).

As the bacterial culture reaches an O.D.600≈2.8, the rate of σ32 synthesis may be increased in response to some form of cellular stress, which in turn initiates transcription of up to ~30 hsp genes and thus increases the cellular concentration of several hsp (Fig. 4.11B).
Fig. 4.11: Schematic representation of the expression of ompT-pbPLA$_2$ induced at low and high cell culture densities. (A) At low induction: (1) protein expression is induced by the addition of IPTG. (2) Due to the high level of expression from the T7 based plasmid, a large amount of protein is produced, but the cellular level of SecB is too low to maintain all the protein in a soluble form. (3) Thus the majority of the protein forms insoluble inclusion bodies of ompT-pbPLA$_2$ in the cytoplasm and only $\sim$20% of the protein expressed is correctly processed (4). (B) When protein expression is induced at a high cell density, a "stress" response is initiated in reply to an environmental change (e.g. reduction in glucose). (1) The stress response is controlled by sigma-32 ($\sigma^{32}$), an alternative RNA polymerase transcription factor which specifically recognises the promoters of several hsp genes. (2) The $\sigma^{32}$-RNA polymerase transcription of the hsp genes causes the elevated production of these proteins. (3) Upon induction of ompT-pbPLA$_2$, the cell cytoplasm is loaded with several hsp which bind the PLA$_2$ polypeptides. The hsp can substitute for SecB, thus maintaining the solubility of ompT-pbPLA$_2$. (4) The hsp are then able to direct the polypeptide to the Sec-machinery via the tertiary complex with SecA. At the Sec YEG complex the polypeptide is translocated into the periplasm. (5) Therefore a greater percentage of the expressed PLA$_2$ is processed and forms insoluble inclusion bodies within the periplasmic space.
Therefore there is now a cellular pool of hsps that are able to prevent the aggregation of the overexpressed ompT-pbPLA2 and compensates for the very low level of SecB present in the cell cytoplasm. It may be that the hsp binds the preprotein to form the polypeptide/hsp complex. The signal sequence of the polypeptide/hsp complex directs the association with SecA. It is this tertiary complex that is able to "recognise" the membrane embedded Sec YEG translocase and translocate the polypeptide into the periplasm. Maintaining the polypeptide in a soluble form prior to translocation is critical to the efficient export of all secreted E. coli proteins (Langer and Neupert, 1986; Leemans et al., 1989), but even more so for ompT-pbPLA2, as otherwise the protein will form insoluble inclusion bodies within the cell cytoplasm. By growing cultures at lower temperatures (e.g. 25-30°C), protein export in bacteria has been shown to be increased (Leemans et al., 1989). This is probably because of a reduction of hydrophobic interactions between the expressed polypeptide chains, or it has been speculated that higher temperatures may favour folding intermediates that aggregate (Leemans, et al., 1989). Thus under these growth conditions, (i.e. low growth temperature and an increase in the levels of hsps), the now solubilised ompT-pbPLA2 can be targeted to the periplasmic space via the Sec complex, where >95% of the protein is properly processed. Once processed the pbPLA2 forms insoluble inclusion bodies within the periplasmic space.

There is substantial evidence that supports the idea that hsps are involved in protein translocation and can substitute for SecB. The most likely hsps which may be able to participate in protein export are DnaK and DnaJ. It has been shown that mutant E. coli strains lacking SecB are able to grow on minimal media but not on rich media. Overexpression of \( \sigma^{32} \) reversed the effects so the cells can now grow on rich media (Altman et al., 1991). Therefore the increased levels of hsps (caused by the overexpression of \( \sigma^{32} \)), could perform the functions of SecB, and thus compensate for its absence. This observation implies that some hsps can substitute for the SecB function by acting as "backup chaperones". The level of hsps in the SecB mutant may be sufficient to permit growth and protein export in minimal media (as the cells are growing slowly), but not under the conditions of rapid growth i.e. with rich media. The effect of deleting SecB can also be reversed by the over-production of DnaJ and DnaK (Wild et al., 1992). The cells are able to grow on rich media and also accelerate the export rate of maltose binding protein to the periplasmic space, thus supporting the theory that
other molecular chaperones, in particular DnaJ and DnaK, can carry out the cellular functions of SecB (Wild et al., 1992). The most convincing evidence for the role of DnaJ and DnaK in protein export comes from experiments carried out with human granulocyte-colony stimulating factor (hG-CSF) (Pérez-Pérez et al., 1995). The secretion of this protein into the periplasmic space of E. coli was shown to be relatively poor, only ~10% of the protein expressed is correctly processed. To improve the percentage of protein which was translocated several different chaperones were individually co-expressed with hG-CSF. When GroEL and GroES were co-expressed with hG-CSF, no significant increase in protein translocation was observed. The same result was obtained with the overexpression of SecB. Only with the overexpression of DnaJ and DnaK was there an increase in the translocation and processing of hG-CSF from ~10% to 60-80% (Pérez-Pérez et al., 1995).

As discussed above, the translocation of a variety of proteins can be significantly improved by the over production of hsps which have been subcloned into vectors under the control of strong promoters. It may be possible to improve the translocation efficiency of secreted recombinant proteins by simply inducing protein expression when the bacterial cells have reached the stationary phase of growth. At this stage of growth, the level of several hsps may be greater due to a cellular stress response. This phenomenon was only seen with bacterial cultures grown on minimal media, but not with cells grown on rich media.

The increased level of translocation and processing of bovine pancreatic phospholipase A₂ brought about by inducing expression at high cell densities had a significant effect on the final amount of refolded and purified recombinant protein isolated per litre of bacterial culture. Prior to this the average amount of enzyme isolated was ~2-5 mg/L of bacterial culture, after induction at high cell densities this was increased to ~20 mg/L.

4.8 In vitro refolding of recombinant PLA₂.

In vitro refolding of polypeptides is a complex process as there are a wide range of parameters that can be varied to achieve the most suitable conditions to allow efficient refolding. The presence of disulphide bonds further complicates in vitro refolding, as the
Chapter 4: Overexpression, refolding and purification of bPLA₂ from E. coli.

Conditions must favour the formation of disulphide bonds as well as the native protein conformation.

PLA₂ is a particularly problematic protein for refolding in vitro due to the large number of disulphides bonds present within such a small enzyme. PLA₂s from a wide range of sources have been successfully overexpressed in E. coli and refolded, but yields generally have been disappointing, as shown in Table 4.4.

Folding from the denatured state is very fast (milliseconds to minutes) for small proteins that do not contain disulphide bonds or in which the native disulphide bonds are intact (Martin et al., 1991). However, when refolding in vitro also requires disulphide bond formation, the renaturation rate is usually much slower (several minutes to hours) and requires the presence of significant concentrations of reductants (Gilbert, 1994). However, disulphide bond formation in vivo occurs within seconds as other enzyme systems (such as protein disulphide isomerase [PDI] in eukaryotes or the Dsb enzyme system in prokaryotes) are present which accelerate the process (Bardwell, 1994). To account for the slow rate of disulphide bond formation (observed in vitro), during oxidative refolding and the requirement of a reducing agent, it has been shown that "kinetic traps" play an important role.

<table>
<thead>
<tr>
<th>Source of PLA₂</th>
<th>Amount of purified protein (mg)/L of bacterial culture</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met 8→Leu human platelet PLA₂</td>
<td>1.1</td>
<td>Franken et al., 1992.</td>
</tr>
<tr>
<td>Honey bee venom PLA₂</td>
<td>8.9</td>
<td>Dudler et al., 1992.</td>
</tr>
<tr>
<td>Cobra venom PLA₂</td>
<td>0.8</td>
<td>Kelly et al., 1992.</td>
</tr>
<tr>
<td>Porcine pancreatic PLA₂</td>
<td>0.3-0.5</td>
<td>Bhat et al., 1993a</td>
</tr>
<tr>
<td>Porcine pancreatic PLA₂</td>
<td>12.8</td>
<td>van den Berg et al., 1995b.</td>
</tr>
<tr>
<td>Bovine pancreatic PLA₂</td>
<td>&lt;5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Noel et al., 1991.</td>
</tr>
</tbody>
</table>

Table 4.4: Levels of recombinant PLA₂ overexpressed in E. coli and refolded in vitro. *The final amount of purified protein was not specified, thus this value is based upon the amount of crude protein after refolding.*
Chapter 4: Overexpression, refolding and purification of bPLA₂ from E. coli.

Kinetic traps are "species from which the generation of native structure and correct disulphide formation are slow" (Gilbert, 1994). Several mechanisms could provide such kinetic traps:

- The generation of the native like structure (or even non-native structure) without disulphide formation could trap cysteine residues in the reduced state so that an unfavourable structural rearrangement must precede chemical oxidation.

- The formation of stable non-native (or native) disulphides could trap the protein, so that disulphide bond reduction (the rate marked * in Fig. 4.5) could be slow for stable disulphides. Therefore, reduction of the disulphide must take place in order to allow for the rearrangement of the disulphides so that the protein eventually arrives at its native structure.

- With intermolecular disulphide reagents such as oxidised glutathione (GSSG), formation of glutathione mixed disulphides with all available cysteines will prevent protein disulphide formation.

Regeneration of disulphide bonds in reduced or S-sulphonated polypeptides is achieved by the use of low molecular weight disulphides and thiols. These systems increase both the rate and the yield of renaturation/re-oxidation by facilitating rapid reshuffling of incorrect disulphide bonds as shown in Fig. 4.5.

Mixtures of oxidised and reduced glutathione, cysteine, cysteamine or β-mercaptoethanol are commonly used at concentrations of 1-5 mM thiol, and 0.01-0.5 mM disulphide agent. This ratio of reduced to oxidised thiol is similar to that observed in vivo (Hwang et al., 1992). The polypeptide prior to refolding is solubilised in high concentrations of denaturants (e.g. 8 M urea or guanidinium chloride). Disulphide bond formation and refolding of the polypeptide to its native structure is achieved by the reduction in concentration of the denaturant, either by dialysis or dilution with refolding buffer. Leaving the protein in a low concentration of denaturant (e.g. 1 M urea) may prevent the formation of
aggregates and allow further reshuffling of incorrectly formed disulphide bonds. This is because the low molecular weight thiols may have access to these bonds due to localised denaturation of the polypeptide.

Air oxidation can also be used to oxidise thiols to form disulphide bonds (Thatcher and Hitchcock, 1994; Jaenicke and Rudolph, 1990). This approach is most effective when the reduced protein can be induced to adopt a near native conformation prior to oxidation. When the reduced protein is unable to form such a stable intermediate, air oxidation can lead to a mixture of misfolded species (Thatcher and Hitchcock, 1994). The absence of low molecular weight thiol can slow the rearrangement of mismatched disulphides to the native structure. Air oxidation is also slow, but can be accelerated by trace amounts of divalent metal ions such as Cu$^{2+}$ (Jaenicke and Rudolph, 1990).

There was a significant improvement in the amount of bPLA$_2$ that can be isolated using the protocol outlined in the Methods section compared with already published data (Table 4.4). The first improvement was from inducing the culture when the cells are nearing the stationary growth phase, which increases translocation efficiency. The second improvement results from refolding the protein in the absence of molecular oxygen. As demonstrated, the presence or absence of oxygen does not affect the percentage of the enzyme that can be activated by trypsin hydrolysis (which is ~40% of the total soluble protein in both cases). Instead, the presence of oxygen causes an increased loss of enzyme from solution during refolding. This may due to molecular oxygen causing oxidation of the S-sulphonated Cys side-chains, thus forming non-native disulphide bonds. This protein conformation may then favour protein aggregation resulting in the precipitation of the protein from solution. Protein which is lost during dialysis against distilled water (prior to trypsin activation) is assumed to be incorrectly folded pbPLA$_2$, as native bPLA$_2$ remains soluble over a wide pH range.

Although 22% of the initial pbPLA$_2$ can be correctly refolded, a further 10-15% of this will be lost during final purification. This may appear to be very low compared to in vivo folding in eukaryotes which appears to be close to 100%. However protein folding in vivo only appears to be 100% successful because eukaryotic cells are very efficient at removing misfolded proteins (Hurtley and Helenius, 1989; Pelham, 1989). Also, in vivo protein folding
Chapter 4: Overexpression, refolding and purification of bPLA₂ from E. coli.

is more efficient as other proteins are involved, for example chaperones (e.g. hsp60 and 70) found within the endoplasmic reticulum (ER) prevent inappropriate association between polypeptide chains which maintains protein solubility, allowing the protein to achieve its native conformation (Langer and Neupert, 1986; Gething and Sambrook, 1992; Hlodan and Hartl, 1994). Proteins with disulphide bonds also require assistance during folding to form the native conformation. This is achieved in eukaryotes by PDI, which is located in the ER and catalyses thiol/disulphide exchange including net oxidation, reduction and rearrangements (Tuie et al., 1994; Gilbert, 1994).

There are several important parameters that seem to significantly affect in vitro refolding of proteins, these are:

- the method of breaking any disulphide bonds formed during isolation of the protein (e.g. β-mercaptoethanol or chemical modification by S-sulphonation).
- the method of refolding (e.g. dialysis, rapid dilution, etc.)
- the concentration and type of redox couples used to reoxidise any disulphide bonds (e.g. oxidised/reduced glutathione or cysteine/cystine).
- the presence of any additives to the refolding buffer (e.g. salts or surfactants)
- the pH of the refolding buffer.
- the temperature at which the refolding is carried out.
- the protein concentration.

Unfortunately, optimal conditions are target protein dependent and so need to be determined empirically for each protein. Some of the effects of these parameters on protein refolding are discussed below.

Isolation of the overexpressed protein from inclusion bodies usually causes the air oxidation of Cys thiols, producing a randomly disulphide bridged protein aggregate. The advantages of S-sulphonation over other methods of breaking these disulphide bonds have been discussed in the Results Section 4.2. Human cathepsin B (a lysosomal cysteine protease), which contains 6 disulphide bonds, forms insoluble inclusion bodies when over-expressed in E. coli (Kuhelj et al., 1995). It was shown that the S-sulphonated polypeptide produced a
greater percentage of correctly refolded protein compared to the dithiothreitol reduced form (Kuhelj et al., 1995). Significant purification of pbPLA₂ (as judged by SDS-PAGE) was achieved by S-sulphonation, where the chemically modified pbPLA₂ was found to be highly soluble in 8 M urea, whilst other species remained insoluble.

Ideally, it would be preferable to refold at a high protein concentration as this makes isolation of the enzyme easier. Unfortunately, as the protein concentration is increased the level of aggregation also increases. This is because folding intermediates are also present at a higher concentration thus increasing the probability of them interacting with one another. This is even more crucial to disulphide bridged proteins as high refolding concentrations may favour intermolecular bonds to form rather than intramolecular ones. Therefore the final protein concentrations are usually kept between 20-100 μg/mL, but this value is dependent on the properties of the protein (e.g. hydrophobicity) and so must be determined experimentally. For example, sPLA₂ (Chapter 3) was found to aggregate during refolding at protein concentrations above ~20 μg/mL, whereas pbPLA₂ remained soluble between 30-50 μg/mL.

The pH and temperature at which refolding is carried out also significantly affects the percentage of protein that achieves the native fold. For disulphide bonded proteins there is a fine balance between stability of the polypeptide and disulphide bond formation. This is because disulphide bond formation requires relatively high pHis, as the bond forming species is the reactive thiolate anion (-S⁻) and for a thiol free in solution the pKₐ is ~8.6, although this may be lower depending on the environment of the Cys amino acid. Thiol-disulphide shuffling takes place almost exclusively at pH 7 and above (Creighton, 1984). Thus the pH of the refolding solution determines the rate of bond formation but at a higher pH the solubility or stability of the protein or folding intermediates may be affected (Gilbert, 1994). As with pH, the temperature at which refolding is carried out can also affect the solubility or stability of the protein or folding intermediates. At lower temperatures hydrophobic effects are reduced as is the rate of folding, which may increase the solubility of the protein or folding intermediates (Mitraki and King, 1989; Creighton, 1994; Xie and Wetlauffer, 1996). It may be possible to further increase the percentage of pbPLA₂ with the native fold, for example, by reducing the temperature to 4°C during dilution of the protein from 8 M to 1 M urea. This would reduce the effects of hydrophobic interactions, thus preventing the aggregation of
pbPLA₂ and allowing further disulphide bond "shuffling" to occur until the native structure had been achieved.

A variety of approaches have been used in the in vitro refolding of proteins. Common to all these approaches is that the polypeptide is solubilised in a high concentration of denaturant, which is then diluted to allow the formation of the native protein structure. The most commonly used methods by which this is achieved is either by dialysis or direct dilution. It has been shown that the rate, at which the denaturant is diluted can significantly increase the final amount of soluble protein and the refolding efficiency. For example, with the refolding of porcine heart fumerase, a greater percentage of active enzyme can be isolated if the concentration of guanidinium chloride is lowered by dialysis rather than by dilution (Kelly and Price, 1991).

After in vitro refolding, the enzyme was activated using trypsin to remove the N-terminal heptapeptide from the refolded protein giving the enzymatically active pbPLA₂. The activity of the protease should also degrade any incorrectly folded pbPLA₂ as only the native enzyme with its compact structure is resistant to the action of trypsin (Franlcen et al., 1992). This provided an ideal method of removing the incorrectly folded forms of pbPLA₂ by degradation. In future, it may be possible to purify the correctly refolded pbPLA₂ prior to activation. If this could be accomplished then the incorrectly refolded protein could be recycled (as it consists of ~60% of the protein sample); i.e. subsequent S-sulphonation and refolding.

Further investigation into methods of increasing the refolding efficiency may prove profitable. This may involve varying the refolding conditions, such as temperature or allowing the refolded protein to undergo further disulphide shuffling in the presence of a higher concentration of urea. Nevertheless, the expression, refolding and purification protocol described herein has been shown to be reliable in producing pure recombinant pbPLA₂ sufficient for kinetic and structural analysis. Production of ~20 mg of PLAA₂ /L of bacterial culture is adequate for the economical production of ¹⁵N and ¹³C labelled protein to be used for nmr in an effort to solve the solution structure of pbPLA₂ and its mutants. As discussed in Chapter 5, the expression and refolding of mutant proteins was also found to be more than
Chapter 4: Overexpression, refolding and purification of bPLA from E. coli.

adequate, although the refolding efficiency of the mutant enzyme will depend on how the substituted amino acids affect the folding pathway of the protein.
Chapter 5: Characterisation of bPLA₂ mutants overexpressed in E. coli and refolded in vitro.

CHAPTER 5

CHARACTERISATION OF BPLA₂ MUTANTS OVEREXPRESSED IN E. coli AND REFOLDED IN VITRO.

5.1 Background.

Although bovine and porcine pancreatic enzymes differ by only 19 amino acids (and one insertion at position 121 in pPLA₂) in their primary amino acid sequence (Dijkstra et al., 1983a) they show significant differences in their catalytic properties and affinity for lipid-water interfaces. The N-terminus, the surface loop region 58-71, and the calcium binding loop (residues 25-42) of the pancreatic PLA₂s have been implicated in the interfacial recognition properties of the enzyme (Dijkstra et al., 1981a; 1981b; Dijkstra et al., 1984). The 58-71 loop region is highly conserved in its amino acid sequence between the two proteins. The only differences in this region are at positions 63 and 71 (Dijkstra et al., 1983b) (Fig. 5.1).

![Fig. 5.1: A comparison of the amino acid sequence of the 58-71 surface loop between bovine and porcine pancreatic PLA₂.](image)

<table>
<thead>
<tr>
<th>Residue No.</th>
<th>BOVINE</th>
<th>PORCINE</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>Leu</td>
<td>Leu</td>
</tr>
<tr>
<td>59</td>
<td>Asp</td>
<td>Asp</td>
</tr>
<tr>
<td>60</td>
<td>Ser</td>
<td>Ser</td>
</tr>
<tr>
<td>61</td>
<td>Cys</td>
<td>Cys</td>
</tr>
<tr>
<td>62</td>
<td>Lys</td>
<td>Lys</td>
</tr>
<tr>
<td>63</td>
<td>Val</td>
<td>Val</td>
</tr>
<tr>
<td>64</td>
<td>Leu</td>
<td>Leu</td>
</tr>
<tr>
<td>65</td>
<td>Val</td>
<td>Val</td>
</tr>
<tr>
<td>66</td>
<td>Asp</td>
<td>Asp</td>
</tr>
<tr>
<td>67</td>
<td>Asn</td>
<td>Asn</td>
</tr>
<tr>
<td>68</td>
<td>Pro</td>
<td>Pro</td>
</tr>
<tr>
<td>69</td>
<td>Tyr</td>
<td>Tyr</td>
</tr>
<tr>
<td>70</td>
<td>Thr</td>
<td>Thr</td>
</tr>
<tr>
<td>71</td>
<td>Asn</td>
<td>Glu</td>
</tr>
</tbody>
</table>

The X-ray crystal structures of the two proteins have been solved (Dijkstra et al., 1983a) and a large (localised) structural difference was observed. The 58-71 loop had a completely different conformation in the two enzymes. Fig. 5.2 shows the structures of the enzymes which are modelled on the X-ray crystal structures. In bPLA₂ residues 58-66 form an α-helical conformation and the remaining residues (67 to 71) form a random coil. In pPLA₂ however, residues 58 to 66 are in a random coil and the residues 67 to 71 form a short 3₁₀ helix (Dijkstra et al., 1983a).
The large conformational change between these two regions was attributed to the single amino acid difference at position 63. It can be seen that Val-63 of bPLA$_2$ is exposed to solvent on the surface of the protein, whereas Phe-63 in the porcine enzyme occupies a hydrophobic pocket in the interior of the enzyme (Fig. 5.2). It is thought that in pPLA$_2$ these hydrophobic interactions stabilise the 58 to 71 loop. Thus the single point mutation Phe-63 to Val (F63V) in pPLA$_2$ was constructed to see if the conformation of this loop was due to Phe-63 (Thunnissen et al., 1993). Fig. 5.3 shows the structure of the porcine mutant F63V, modelled on the solved X-ray crystal structure of the enzyme. It can be clearly seen that Val-63 has now oriented itself towards the exterior of the protein and that the 58 to 71 loop region has a conformation very similar to native bPLA$_2$. The possibility of crystal packing interactions causing the conformational change in this region of the protein were discounted since the structures of two crystals with two different space groups were similar.

**Fig. 5.3:** X-ray crystal structure of F63V pPLA$_2$. The mutated residue Phe$\rightarrow$Val-63 is also shown (Thunnissen et al., 1993).
Chapter 5: Characterisation of bPLA₂ mutants overexpressed in E. coli and refolded in vitro.

The effects of the mutation on the activity of the enzyme were also investigated: pPLA₂ has a much higher affinity for the lipid-water interfaces of aggregated substrates (i.e. micelles) compared to bPLA₂. The loop region (58 to 71) is located on the surface of the protein and is part of the interfacial recognition site (IRS). Thus it was of interest to see how the large conformational changes that occurred as a result of the F63V mutation affected binding and activity on micelles. It was shown that the affinity for zwitterionic interfaces (i.e. 1,2-dioctanoyl-sn-glycero-3-phosphocholine) was intermediate between the porcine and bovine enzymes, but the mutant still bound to micelles of n-hexadecanoylphosphocholine (a non-hydrolysable phospholipid analogue) more tightly than bPLA₂ (by an order of magnitude).

Thus it was concluded that although the structure of the loop region of F63V pPLA₂ is similar to bPLA₂, its affinity for the micellar substrate analogue and its enzymatic activity are more like those of pPLA₂. Therefore residue 63 is not primarily responsible for the differences in the binding properties and enzymatic activity seen for the two pancreatic enzymes. Thus the difference in enzymatic/binding properties must be due to one or more of the remaining substitutions.

It seemed more logical to try the reciprocal experiment (i.e. substitute Val-63 for Phe-63 in bPLA₂) since the bovine enzyme has a significantly lower affinity for lipid-water interfaces than the porcine protein and any bovine mutants which had porcine-like properties would be easily identified. Site-directed mutagenesis was used to investigate what contribution residues 63 and 71 in the 58-71 surface loop make to interfacial recognition (residue 71 being the only other difference in this region between the two enzymes). This would also allow us to investigate whether these residues were involved in the binding and kinetic differences seen between bovine and porcine pancreatic PLA₂. The three mutant proteins made were: Val-63→Phe-63 (V63F), Asn-71→Glu-71 (N71E) and the double mutant V63FN71E.
Chapter 5: Characterisation of bPLA\textsubscript{2} mutants overexpressed in E. coli and refolded in vitro.

RESULTS AND DISCUSSION

5.2 Molecular biology and mutagenesis of bPLA\textsubscript{2}.

All the cloning and mutagenesis procedures were carried out in the pET 12a expression vector. All mutants were constructed using a PCR based mutagenesis method (see Methods). The codon usage was also changed at the 3' end of the mutant genes; Lys-121 codon was changed from AAA to AAC, as shown in Fig. 5.4.

During the construction of the synthetic wild type gene (Mallinder et al., unpublished work), a high frequency of frame shift mutations were observed involving the deletion of an adenine base in the Lys 120-121 coding sequence. This deletion could be due to sequence slippage errors at the DNA replication point by Taq DNA polymerase during PCR. Deletions or additions during replication (\textit{in vivo}) are thought to result from displacement ("looping-out") of bases from either the template strand (giving a deletion) or the growing strand (giving an addition). These mistakes occur particularly in regions of the DNA sequence that contain stretches of identical bases where the looped-out structure can be stabilised by normal base pairing beyond the unpaired base (see Fig. 5.5). When the miscopied DNA strand acts as a template, the deletion or addition is usually copied accurately, fixing the mutation (Watson et al., 1990). Since Taq DNA polymerase does not have the 3'→5' proof-reading exonuclease activity the frequency for errors will be much greater than \textit{in vivo}.

After carrying out the final PCR to produce the full length product, the isolated DNA was digested and ligated into the pET vector. A second PCR method was used to screen for vector carrying the insert DNA. This allowed the efficient screening of a large number of colonies. The frequency of ligation of the gene into the vector was found to be about 50% and all the positive clones contained the mutagenic oligonucleotide sequence as determined by \textsuperscript{35}S dideoxynucleotide DNA sequencing. Sequencing of putative mutant clones was initially carried out using a specific base termination reaction (see Fig. 5.6). For example if the mutation caused the DNA sequence to change from CTT→TAG then sequencing using only the thymine (T) base-termination mix would be carried out since this would identify the sequence change. By running out the sequence reaction mix of the mutant clones next to the
Chapter 5: Characterisation of bPLA₂ mutants overexpressed in E. coli and refolded in vitro.

Wild type sequence positive clones could easily be identified (Fig. 5.6). Both DNA strands of the gene were then sequenced to confirm that no further mutations had occurred as a result of the PCR.

![Diagram showing codon usage change](image)

**Fig. 5.4:** Codon usage change (bold type) introduced into bovine PLA₂ and its mutants by PCR.

![Diagram showing possible mechanism for high mutation frequency](image)

**Fig. 5.5:** Possible mechanism for the high mutation frequency observed at the 3'-end of the synthetic bovine gene (Watson *et al.*, 1990).
Chapter 5: Characterisation of hPLA₂ mutants overexpressed in E. coli and refolded in vitro.

5.3 Mutant protein expression, refolding and purification.

The recombinant mutant proteins were expressed in the E. coli strain BL21 (DE3) pLysS. The same level of translocation of the mutant proteins occurred as observed for the recombinant wild type protein as judged by SDS-PAGE. Once the inclusion bodies were isolated and S-sulphonated, the protein was refolded in vitro and purified using the same procedures used to purify the recombinant wild type enzyme (Chapter 2, Section 2.5). Fig. 5.7 shows a Tris-Tricine gel of the expression and purification of the double mutant V63FN71E.
Chapter 5: Characterisation of bPLA<sub>2</sub> mutants overexpressed in E. coli and refolded in vitro.

![Image](image-url)

**Fig. 5.7**: 15% Tris-Tricine SDS-PAGE gel of the isolation and purification of the double mutant V63FN71E. The lanes marked are: (1) Triton X-100 supernatant wash 1, (2) Triton X-100 supernatant wash 1, (3) insoluble protein after S-sulphonation, (4)-(5) 0.3% acetic acid precipitated protein (two different loadings), (6)-(7) purified refolded protein, (8) authentic bPLA<sub>2</sub>, and (9) SDS PAGE molecular weight markers.

The level of protein expression and therefore the amount of S-sulphonated polypeptide isolated seemed to vary considerably. This may have been due to differences in the O.D.<sub>600</sub> at which the bacterial cells were induced to express PLA<sub>2</sub> and the length of time the cultures were then left to grow. For example, cells expressing <sup>15</sup>N bPLA<sub>2</sub> (Table 5.1) were induced at O.D.<sub>600</sub> = 2.2 and left for ~12 hours, whereas cultures expressing <sup>15</sup>N V63F were induced at a higher O.D.<sub>600</sub> of 3.0 and then incubated for a further ~18 hours. The amount of S-sulphonated protein isolated from the two cultures was significantly different (257 mg compared with 62 mg per litre of bacterial culture for bPLA<sub>2</sub> and V63F respectively). There may be several reasons for such differences in protein expression levels. Once the bacterial cell density has reached O.D.<sub>600</sub> ~3.0, the media will have become nutrient limited (in this case glucose limited) and the cells will be nearing the stationary growth phase. The majority of the carbon source may have been utilised by the cells for energy and biomass production, and so the availability of carbon compounds that could be used in protein synthesis will be very low. Incubation of the cultures for such long periods of time after induction may lead to
Chapter 5: Characterisation of bPLA$_2$ mutants overexpressed in E. coli and refolded in vitro.

the degradation of the recombinant protein. What does seem clear is that there appears to be only a small "window" of time within which protein expression can be initiated to produce the greatest amount of protein (between O.D$_{600}$ of 2.2-2.8). Induction of protein expression below O.D$_{600}$ ~2.0 favours the formation of the unprocessed OmpT polypeptide species which cannot be trypsin activated, whereas induction at or above O.D$_{600}$ ~3.0 reduced the level of protein expressed. Therefore the final amount of enzyme refolded per litre of bacterial culture varied considerably depending on the O.D$_{600}$ value at which the protein was induced (from ~7 to 22 mg/L of culture). As a result of this the exact amount of S-sulphonated PLA$_2$ used in each refolding cannot be determined and this in turn means that the folding efficiency can only be estimated (see Table 5.1). It was assumed that ~70% of the S-sulphonated protein to be refolded was PLA$_2$, (as judged by SDS-PAGE lanes 3 and 4 of Fig. 5.7), and that only 40% of the soluble protein eluted from the Fast Flow S-Sepharose column could be activated (as determined using the egg yolk lecithin/deoxycholate mixed micelle assay). Therefore the refolding efficiency of N71E (~18%) was similar to that of the wild type enzyme (~22%) (Chapter 4, Section 4.3), showing that this amino acid substitution did not have a deleterious effect on the folding pathway. This was not the case for the double mutant V63FN71E where the folding efficiency was approximately a third (~8%) of that of the wild-type enzyme. Table 5.1 shows the final amounts of recombinant protein isolated from E. coli. V63F could not be compared directly as this sample was prepared prior to the discovery that induction of protein expression at higher O.D$_{600}$ values resulted in an increase in the translocation efficiency.

The purification procedures used to isolate the mutants were identical to those used for recombinant bPLA$_2$ (Chapter 2, Sections 2.5.2 to 2.5.5). The elution characteristics of all the mutants from the Phenyl Sepharose and High Load S-Sepharose columns were similar to bPLA$_2$. 

151
Chapter 5: Characterisation of bPLA₂ mutants overexpressed in E. coli and refolded in vitro.

<table>
<thead>
<tr>
<th></th>
<th>15N Wild type (mg)</th>
<th>15N V63F (mg)</th>
<th>N71E (mg)</th>
<th>V63FN71E (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-sulphonated protein refolded</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Soluble protein eluted from the Fast Flow S-Sepharose column, prior to trypsin activation</td>
<td>47</td>
<td>103</td>
<td>62</td>
<td>29.5</td>
</tr>
<tr>
<td>Protein eluted from the Phenyl Sepharose XK 26/10</td>
<td>19.1</td>
<td>44.3</td>
<td>23.8</td>
<td>9.7</td>
</tr>
<tr>
<td>Pooled fractions shown to be pure enzyme using the Mono P 5/20 column</td>
<td>12.2</td>
<td>22.3</td>
<td>19.1</td>
<td>7.9</td>
</tr>
<tr>
<td>Folding efficiency (%)</td>
<td>13.6</td>
<td>n</td>
<td>17.9</td>
<td>8.4</td>
</tr>
<tr>
<td>Amount of enzyme per L of bacterial culture</td>
<td>15.7</td>
<td>6.9⁸</td>
<td>22.1</td>
<td>12.8</td>
</tr>
</tbody>
</table>

Table 5.1: Refolding yields of recombinant PLA₂ expressed in E. coli. The refolding conditions used are outlined in Chapter 2, Section 2.5. Approximately 200 mg of S-sulphonated protein was used per refolding.

⁸See Section 5.3 for an explanation of the overall low yield.

5.4 Protein concentration determination using the bicinchoninic acid assay.

The protein concentration was routinely determined using the A₂₈₀ and the extinction coefficient for PLA₂ (using the Beer-Lambert equation A = εCl, where A = A₂₈₀; C = molar concentration of the sample; ε = molar extinction coefficient (13,500 M⁻¹ cm⁻¹ for PLA₂) and l = length of the light path through the sample) as it was the simple, quick and accurate. Therefore in order for the protein concentrations of the mutants to determined by measuring the A₂₈₀, it was necessary to verify that the point mutations had not significantly altered the extinction coefficient (ε) of the protein. This was accomplished using the bicinchoninic acid protein assay (BCA). A standard curve was constructed using authentic bPLA₂. This allowed the observed absorbance measured using the BCA assay to be correlated back to an actual protein amount. From the assay, the dependence of protein concentration on absorbance determined for each mutant was very similar showing that the amino acid substitutions did not affect the extinction coefficient significantly.
5.5 Electrospray mass spectrometry.

Electrospray mass spectrometry (EMS) was used to determine the exact molecular mass of the mutant proteins. This would also confirm that the expressed and refolded proteins contained the required mutation(s) as there should be a change in their mass relative to the wild type. An example of the analysed spectra obtained for V63F is shown in Fig. 5.8.

![Electrospray mass spectrum of V63F](image)

**Fig. 5.8:** Transformed electrospray mass spectrum of V63F. The calculated mass was 13,829.54 Da and the observed mass was found to be 13,830.82 ± 1 Da.

The calculated molecular mass of the mutant was 13,829.54 Da and the observed mass was found to be 13,830.82 ± 1 Da. From the spectra acquired, other species were seen to be present whose masses were multiples of 99 Da greater than that of the mutant producing peaks at masses of 13,930 and 14,030 Da. This has been observed previously and attributed to adducts with phosphoric or sulphuric acid (Di Marco *et al.*, 1992). The higher molecular mass species observed for V63F (Fig. 5.8) may have been due to trapped phosphoric acid ($H_3PO_4$, $M_r = 98$ Da) within the enzyme molecule (Fountoulakis *et al.*, 1995), thus producing the observed protein heterogeneity. The most likely source of the phosphoric acid would be from the oxidised glutathione solution, as 200 mM (NH$_4$)$_2$HPO$_4$ was used to buffer the solution during the oxidation of the reduced glutathione (Appendix A5.2). Protein heterogeneity has
been observed in the isolation of soluble interferon γ receptor overexpressed in *E. coli* (Fountoulakis *et al.*, 1995). In this instance the trapped phosphoric acid produced a heterogeneous protein sample, which had full biological activity, but gave one to three multiples of 97 ± 2 Da in the spectra from ion spray mass spectrometry. Gas chromatography-mass spectrometry analysis revealed the presence of phosphoric acid in the protein sample (Fountoulakis *et al.*, 1995).

Table 5.2 lists the expected and actual molecular masses measured by EMS. EMS allowed the exact molecular mass (± 1 Da) of the various enzymes to be determined. This technique also demonstrated that the percentage incorporation of isotopic label (15N) was > 99.9% which was reflected in the increased mass of the proteins. Finally, since the observed and the expected molecular masses were very similar this suggests that refolding the protein using high concentrations of urea as the denaturant did not result in chemical modification of the enzyme.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Calculated mass (Da)</th>
<th>Experimentally determined mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15N wild type</td>
<td>13945.49*</td>
<td>13944.98 ± 1.5</td>
</tr>
<tr>
<td>15N V63F</td>
<td>13993.54*</td>
<td>13992.47 ± 0.6</td>
</tr>
<tr>
<td>V63F</td>
<td>13829.54</td>
<td>13830.82 ± 1.0</td>
</tr>
<tr>
<td>N71E</td>
<td>13796.51</td>
<td>13798.03 ± 0.5</td>
</tr>
<tr>
<td>V63FN71E</td>
<td>13844.56</td>
<td>13844.81 ± 0.7</td>
</tr>
</tbody>
</table>

Table 5.2: EMS determined molecular masses of 15N labelled and mutant proteins of bPLA2. The calculated masses marked (*) were determined assuming 100% incorporation of 15N.

5.6 Elution characteristics from the Mono P 5/20 chromatofocusing column.

The Mono P (chromatofocusing) column separates proteins on the basis of their isoelectric points (pI). To investigate whether the mutations introduced into bPLA2 had changed the net charge of the protein (i.e. the pI), the mutant enzymes were loaded and eluted from the Mono P chromatofocusing column. Fig. 5.10 shows the elution profiles of the three mutant and native bovine PLA2s from the Mono P column. The pH gradient was produced by
Chapter 5: Characterisation of bPLA₂ mutants overexpressed in E. coli and refolded in vitro.

the Polybuffer 96/acetic acid pH 6.0 buffer, to give a linear pH range from 9.6 to 6.0. The measured pIs as determined from the Mono P column are shown in Table 5.3. V63F and authentic bPLA₂ had identical pI values of 8.5, which is not surprising since the mutation involved the substitution of one hydrophobic residue for another (i.e. Val→Phe). As expected the introduction of a negatively charged residue (Asn→Glu) in the N71E mutant significantly lowered the pI of the protein from 8.5 to ~7.9. The V63FN71E mutant also had a similar pI to N71E of ~8.0, but as can be seen from Fig. 5.10 the double mutant has a slightly higher pI and so can be separated from N71E.

Table 5.3: The pIs of authentic and mutant recombinant bPLA₂, as determined from the elution of the enzymes from the Mono P 5/20.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authentic bPLA₂</td>
<td>8.5</td>
</tr>
<tr>
<td>V63F</td>
<td>8.5</td>
</tr>
<tr>
<td>N71E</td>
<td>7.9</td>
</tr>
<tr>
<td>V63FN71E</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Fig. 5.10: Elution profile of the separation of the mixture of the four bovine proteins using the Mono P 5/20 column. Approximately 30 μg of each protein was used. The pH gradient was produced by Polybuffer 96 pH 6.0 with acetic acid.
Chapter 5: Characterisation of bPLA2 mutants overexpressed in E. coli and refolded in vitro.

5.7 Nmr of wild type and V63F PLA2.

The reasons for determining the nmr solution structure of the recombinant bPLA2 and the V63F mutant were: i) to verify the conformation of the 58-71 surface loop and ii) to ascertain which residues may be responsible for the increased affinity of the V63F bPLA2 mutant for lipid-water interfaces (see Chapter 6, Section 6.9). It was hoped that nmr would allow the solution structure of both the bPLA2 and V63F enzymes to be determined and so clarify the actual conformation of the 58-71 surface region and its role in interfacial recognition. The nmr experiments were carried out by Dr. P. Kilby and Dr. W. U. Primrose (Biological NMR Centre, University of Leicester). A 2D $^1$H-$^1$H total correlation spectrum (TOCSY) of the V63F mutant was recorded and compared to that of authentic bPLA2 (Fig. 5.11 of the aliphatic region and Fig. 5.12 of the aromatic region). The cross-peak arrangement in the aliphatic and aromatic regions of the nmr spectra are very similar for authentic and V63F bPLA2. This suggests that the substitution of the Val→Phe-63 residue does not significantly perturb the proteins tertiary structure. From Fig. 5.11a it can be seen that an assigned cross-peak from Val-63 is present (positioned between Val-65 and Val-109) in the wild type spectra but is absent from the aliphatic region of the V63F mutant. However, new resonances from the substituted Phe-63 appear in the aromatic region of the nmr spectra (Fig 5.12b). These new resonances for Phe-63 (at 7.3 and 7.4 ppm) are in a similar position to the chemical shifts for Phe-63 that appear in the porcine enzyme (Dekker et al., 1991). Therefore the nmr experiments confirmed the presence of the V63F substitution.

From the nmr spectra of the protein (Fig. 5.11 and 5.12) it can be seen that there is no significant change in the secondary structure. This does not mean that the surface loop has not changed its conformation as this region has been difficult to assign as the amide protons undergo rapid exchange making the protons "invisible" to nmr thus giving few nuclear Overhauser effects (NOEs). The NOE relies on the interactions that occur through space and can therefore provide information on nuclei which are adjacent in space but not linked through bonds. The rapid exchange of amide protons could reflect the flexible nature of the backbone within particular regions of the protein and hence the lack of ordered secondary structure. Rapid proton exchange has also been observed in the assignment of the structure of
Chapter 5: Characterisation of bPLA₂ mutants overexpressed in E. coli and refolded in vitro.

Porcine pancreatic PLA₂ free in solution (Peters et al., 1992; van den Berg et al., 1995a; 1995b).

Full assignment of both the recombinant wild-type and V63F bPLA₂s still remains to be completed. Isotopic labelling of the proteins may be required to assign regions of signal overlap or to identify specific residues. Regions of the enzyme that undergo rapid amide exchange (for example the first few residues of the N-terminal helix and the 58-71 region) may be easier to assign in the ternary complex (i.e. when the protein is bound to a detergent micelle with a non-hydrolysable ligand bound at the active site) because these mobile regions may become more ordered in the ternary complex, thus reducing the amide exchange rates. This has been seen for pPLA₂ (van den Berg et al., 1995a; 1995b; 1995c). The ternary nmr structure of pPLA₂ was found to be similar to the X-ray crystal structure. Residues 62-68 were poorly defined and this was attributed to the lack of long-range NOEs reflecting the high flexibility within this region. However, residues 69-72 were found to be ordered due to interactions with the N-terminal helix. Generally, the 58-71 surface loop in the ternary structure of pPLA₂ was similar to the X-ray crystal structure, with the loop pointing inwards (van den Berg et al., 1995a; 1995b).
Fig. 5.11: Part of the aliphatic region from a 2D-TOCSY spectrum of (a) 0.5 mM authentic bPLA$_2$ and (b) 0.2 mM V63F. Nmr conditions: 20 mM d$_4$ succinate "pH 6.0", 50 mM calcium chloride and 125 mM sodium chloride at 314K.
Fig. 5.12: Part of the aromatic region from a 2D-TOCSY spectrum of (a) 0.5 mM authentic bPLA2 and (b) 0.2 mM V63F. Nmr conditions: 20 mM d4 succinate pH 6.0, 50 mM calcium chloride and 125 mM sodium chloride at 314 K.
6.1 Determination of critical micelle concentrations of substrates.

The physical aggregated state of the substrate plays a key role in the kinetic characteristics of \( \text{PLA}_2 \). \( \text{PLA}_2 \) shows a marked increase in catalytic rate once the substrate has formed aggregated structures such as micelles. This can most easily be observed with short-chain phospholipids, which have relatively high cmc values (Roberts, 1988). Therefore the aggregated state of a variety of phospholipid substrates and lysophospholipid analogues was investigated by the 8-anilino-1-naphthalenesulphonic acid (ANS) fluorescence technique (de Vendittis et al., 1981). The fluorophore ANS, when free in solution, has a negligible fluorescence, but there is a large increase in its fluorescence when the molecule is in a hydrophobic environment. The surfactant is titrated into a cuvette containing ANS only. The transition from monomer to organised aggregated structures such as micelles or vesicles is accompanied by a large increase in the fluorescence of ANS. This is due to the hydrophobic acyl chains of the ligand enveloping the fluorophore, thus changing its local environment. By plotting the fluorescence increase against the ligand concentration two linear lines are produced, the intersection of which gives the critical micellar concentration (cmc) i.e. the concentration of ligand at which micelles or larger aggregated structures form. Fig. 6.1 shows an actual titration curve for the cmc determination of \( n \)-hexadecylphosphocholine (\( \text{C}_{16}\text{PN} \)); from the plot the cmc was found to be 4.8 \( \mu \text{M} \). The cmc of the ligands listed in (Table 6.1) were measured under the conditions used in the actual assays.
Chapter 6: Kinetic characterisation of bPLA₂ mutants.

Fig. 6.1: Titration curve for the cmc determination of CigPN. The cmc was determined from the intersection of the lines and was found to be 4.8 μM. Assay conditions: were 50 μM ANS, 100 mM sodium chloride, 50 mM calcium chloride, 100 mM sodium acetate pH 5.0 at 25°C. Excitation wavelength was at 295 nm and the emission measured at 340 nm. Excitation and emission slits were set at 5 nm.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Measured cmc (μM)</th>
<th>Published value (μM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₆PN</td>
<td>4.8</td>
<td>10</td>
<td>(Weltzien, 1979; de Araujo et al., 1979)</td>
</tr>
<tr>
<td>HEPC</td>
<td>5.0</td>
<td>5.0</td>
<td>(Aarsman and van den Bosch, 1979)</td>
</tr>
<tr>
<td>diC₄PC</td>
<td>185</td>
<td>170</td>
<td>(Wells, 1974)</td>
</tr>
<tr>
<td>diC₆PC</td>
<td>7500</td>
<td>9800</td>
<td>(Wells, 1974)</td>
</tr>
</tbody>
</table>

Table 6.1: Cmc values determined using the ANS fluorescence method for a variety of surfactants.

The cmc values measured were found to be similar to those stated in the literature. The cmc is dependent on the assay conditions (such as salt concentration and temperature) and this may account for the differences seen for C₁₆PN and diC₆PC.
Chapter 6: Kinetic characterisation of bPLA₂ mutants.

6.2 Binding of PLA₂ to micelles of C₁₆PN measured by fluorescence spectroscopy.

The affinity of each enzyme for surfactant-water interfaces was measured using the non-hydrolysable lysophospholipid analogue C₁₆PN. C₁₆PN forms zwitterionic micelles above its cmc, and was measured to be 4.8 μM. The fluorescence from the unique tryptophan at position 3 in PLA₂ was used to monitor the localised environment of the protein during the titration. The fluorescence data were corrected and analysed as described in the Methods section. The data were fitted using the single binding site equation provided with the Grafit package, which gave the value of \( N \cdot K_d \). The \( N \cdot K_d \) represents the value where 50% of the enzyme is saturated by micelles, \( N \) being the number of monomers in the micelle and \( K_d \) the dissociation constant. Fig. 6.2 shows the result of a typical titration curve after fluorescence correction (as outlined in the Methods) and computer fitting.

It was found that the results of this experiment were not reproducible if other methods of mixing the assay mixture were attempted, such as mixing \textit{in situ} with a stirring rod. Thus continuous stirring with a magnetic bar was used throughout the experiment. The experiments were carried out at pH 5.0 so that the enzyme would be positively charged and therefore would have a high affinity for the surfactant interface. At pH 5, the measurement of the binding of PLA₂ is reproducible, whereas at pH 6 or higher it is not (Verheij, personal communication). The results of the C₁₆PN titrations with the series of enzymes is shown in Table 6.2.

From Table 6.2 it can be seen that the porcine enzyme has a ~20 fold greater affinity for the zwitterionic interface than the bovine enzyme. The introduction of the Val-63→Phe-63 mutation into the bovine enzyme has improved its binding properties, but not to that of the porcine enzyme. N71E shows the greatest difference in binding to micelles of C₁₆PN, with a five-fold increase in the \( N \cdot K_d \) compared to the native bovine protein. Thus the introduction of a negatively charged side chain significantly reduces the proteins affinity for zwitterionic micelles. The deleterious effect of the glutamate introduced at position 71 seems to be compensated by the presence of the phenylalanine in the double mutant (V63FN71E) since the \( N \cdot K_d \) value for this mutant is similar to that of native bovine enzyme.
Chapter 6: Kinetic characterisation of bPLA₂ mutants.

Fig. 6.2: Computer fitted titration curve of C₁₆PN with V₆₃F₇₁E. Assay conditions: 5 μM protein, 100 mM sodium chloride, 50 mM calcium chloride, 100 mM sodium acetate pH 5.0 at 25°C. Excitation was at 295 nm and the emission measured at 340 nm. Excitation and emission slits widths were set at 5 nm.

Table 6.2: N-K₄ values for the titration of C₁₆PN with various PLA₂s. Experimental conditions: 5 μM enzyme, 100 mM sodium chloride, 50 mM calcium chloride, 100 mM sodium acetate pH 5.0 at 25°C.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>N-K₄ (mM)</th>
<th>Relative to wild type bPLA₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>bPLA₂</td>
<td>2.57 ± 0.04</td>
<td>1.00</td>
</tr>
<tr>
<td>pPLA₂</td>
<td>0.116 ± 0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>V₆₃F</td>
<td>0.42 ± 0.02</td>
<td>0.17</td>
</tr>
<tr>
<td>N₇₁E</td>
<td>12.88 ± 0.45</td>
<td>5.01</td>
</tr>
<tr>
<td>V₆₃F₇₁E</td>
<td>2.96 ± 0.09</td>
<td>1.15</td>
</tr>
</tbody>
</table>

In order to discount the possibility that Glu-71 was not fully ionised at pH 5.0 the titrations were repeated at pH 8.0. The results are shown in Table 6.3.
Chapter 6: Kinetic characterisation of bPLA₂ mutants.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$N\cdot K_d$ (mM)</th>
<th>Relative to wild type bPLA₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>bPLA₂</td>
<td>2.66 ± 0.08</td>
<td>1.00</td>
</tr>
<tr>
<td>pPLA₂</td>
<td>0.165 ± 0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>V63F</td>
<td>0.093 ± 0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>N71E</td>
<td>17.11 ± 1.40</td>
<td>6.40</td>
</tr>
<tr>
<td>V63FN71E</td>
<td>8.67 ± 0.18</td>
<td>3.30</td>
</tr>
</tbody>
</table>

Table 6.3: $N\cdot K_d$ values for the titration of C₁₆PN with various PLA₂s. Experimental conditions: 5 μM enzyme, 100 mM sodium chloride, 50 mM calcium chloride, 100 mM Tris pH 8.0 at 25°C.

At pH 8.0 the affinity constants of both native bovine and porcine PLA₂ for C₁₆PN micelles are similar to those measured at pH 5.0. The increase in pH has further decreased the $N\cdot K_d$ of V63F, so that its binding is now more similar to the porcine than the bovine enzyme at this pH. The affinity of N71E for the interface is further reduced, but now the presence of the Val→Phe in the double mutant can no longer fully compensate for the presence of Glu-71. The affinity for micelles of the double mutant is now ~3 fold less than bPLA₂.

Emission scans of the various enzymes before and after the titration all show blue shifts of between 5-10 nm and an enhancement in the fluorescence emission of Trp-3 (for example see Fig. 6.3). This indicates that Trp-3 has moved from a solvent accessible aqueous environment to a less polar environment in the micelle complex.
Fluorescence Arb. units

V63F+C_{16}PN

V63F

Fig. 6.3: The emission scans of V63F in the absence (—) and presence (—) of C_{16}PN. The observed blue shift (~10 nm) of the emission peak is also shown (•••). Assay conditions: 5 μM protein, 100 mM sodium chloride, 50 mM calcium chloride, 100 mM sodium acetate pH 5.0 at 25°C. The sample was excited at 295 nm and the fluorescence emission was measured from 310-450 nm. Excitation and emission slit widths were set at 5 nm.

6.3 The egg yolk lecithin/deoxycholate mixed micelle assay.

The egg-lecithin assay was routinely used to assay for PLA\textsubscript{2} activity using the pH-stat. Because the egg yolk substrate is a complex mixture of lipids, proteins and carbohydrates, it can only be used to measure the relative activity of each enzyme. Egg yolk consists mainly of long-chain ethanolamine and choline phospholipids. Deoxycholate is required to disperse the substrate so that mixed micelles are formed and also to introduce a net negative charge so that PLA\textsubscript{2} will have high affinity for the lipid-water interface. The activity is measured indirectly by the pH-stat. As the enzyme turns over, one proton is released per phospholipid molecule hydrolysed. Since the assay solution has only a slight buffering capacity, the pH of the mixture is reduced by the liberated protons. The pH-stat maintains the pH at 8.0 by titrating a weak solution of NaOH (1-20 mM) into the reaction vessel. Thus by titrating the protons released using a solution of base, the amount of phospholipid hydrolysed can be indirectly measured (Fig. 6.4). The results of the egg-lecithin assay are given in Table 6.4.
Fig. 6.4: Schematic representation of the reaction progress curve from the pH stat. From the initial rate of addition of base (µL/min), the specific activity can be calculated as µmoles base (or H⁺)/min/mg of enzyme.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity (µmoles H⁺/min/mg)</th>
<th>$k_{cat}$ (sec⁻¹)</th>
<th>Relative to wild type bPLA₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>bPLA₂</td>
<td>182.5 ± 1.5</td>
<td>41.1 ± 0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>pPLA₂</td>
<td>705.8 ± 0.2</td>
<td>159.0 ± 0.1</td>
<td>3.9</td>
</tr>
<tr>
<td>V63F</td>
<td>231.0 ± 2.0</td>
<td>52.0 ± 0.5</td>
<td>1.3</td>
</tr>
<tr>
<td>N71E</td>
<td>74.0 ± 2.5</td>
<td>16.7 ± 4</td>
<td>0.4</td>
</tr>
<tr>
<td>V63FN71E</td>
<td>185.3 ± 1.8</td>
<td>41.7 ± 0.4</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 6.4: The results from the egg lecithin assay using the pH stat. Assay conditions: 1 µg (75 pmoles) of enzyme, 2 mL of egg-mix containing 25 mM calcium chloride at 45°C. 5 mM sodium hydroxide was used as the titrant.

Porcine PLA₂ shows the greatest activity on the mixed micelles, four times greater than the bovine enzyme. The mutants show a wide range of activities: V63F shows a 30% increase in activity over that of the native bovine enzyme, whereas N71E displays a 60% reduction. The double mutant V63FN71E has similar activity to that of bovine PLA₂, thus suggesting that the introduction of phenylalanine at position 63 can compensate for the loss of activity caused by glutamic acid at position 71.
6.4 $K_m^{\text{PP}}$ and $k_{\text{cat}}$ determination using diC₈PC micelles.

1,2-dioctanoyl-sn-glycero-3-phosphocholine (diC₈PC) is widely used in the determination of kinetic parameters of PLA₂. Due to the short acyl chain length, this synthetic phospholipid forms micelles and has a cmc of 0.185 mM (as measured using ANS fluorescence). Bovine PLA₂ shows a high affinity for this substrate, making it ideal for characterising the kinetic properties of the mutant proteins. The assays were carried out using the pH-stat and the stock lipid solution was kept at 45°C to prevent the phase transition of the phospholipid which occurs at lower temperatures. Calcium chloride was also left out of the stock solution as its presence caused the fusion of the micelles into larger aggregated structures. In this assay, PLA₂ is hydrolysing substrate which has aggregated into micelles. Therefore, when the enzyme is at the interface it is difficult to distinguish between enzyme binding to the lipid-water interface or active site binding of the substrate, thus the $K_m$ is referred to as $K_m^{\text{PP}}$ and reflects the overall process that occurs at the interface. Thus, determining whether an observed altered activity is the result of changed interfacial binding or kinetic processing is difficult.

Fig. 6.5 shows the results of a typical titration experiment. The concentration of free monomer (0.185 mM) was subtracted to give the concentration of micellar substrate. The activity on monomer was too low to measure and thus the initial rate did not need to be corrected.

Again the results in Table 6.5 show that the introduction of the phenylalanine mutation into b PLA₂ has increased its binding and catalysis compared to that of the bovine protein. Porcine PLA₂ has a similar affinity for the diC₈PC lipid water interface as that of bPLA₂, yet the enzyme has a significantly lower rate of catalysis showing that this is not the preferred substrate for the enzyme. The largest difference in micelle binding and catalysis is for the N71E and the double mutant V63FN71E. The Glu-71 mutation decreased the affinity for the interface, as reflected by the large $K_m^{\text{PP}}$ value, but it also appears to affect $k_{\text{cat}}$. 
Chapter 6: Kinetic characterisation of bPLA$_2$ mutants.

Fig. 6.5: Computer fitted data for the initial rate of hydrolysis of diC$_{24}$PC by V63F. The calculated $K_m^\text{app}$ and $V_{\text{max}}$ are shown. The free monomer concentration (0.185 mM) was subtracted from the total phospholipid concentration. Assay conditions: 0.2 µg (15 pmol) of enzyme, 25 mM calcium chloride, 100 mM sodium chloride and 1 mM sodium tetraborate pH 8.0 at 45°C. The sodium hydroxide concentration used as the titrant was 10 mM.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity (µmoles H$^+$/min/mg)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m^\text{app}$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bPLA$_2$</td>
<td>2715 ± 103</td>
<td>610.9 ± 23</td>
<td>0.62 ± 0.08</td>
<td>9.8 x 10$^5$</td>
</tr>
<tr>
<td>pPLA$_2$</td>
<td>1709 ± 68</td>
<td>404 ± 16</td>
<td>0.50 ± 0.06</td>
<td>8.1 x 10$^5$</td>
</tr>
<tr>
<td>V63F</td>
<td>3175 ± 54</td>
<td>714 ± 12</td>
<td>0.30 ± 0.02</td>
<td>23.8 x 10$^5$</td>
</tr>
<tr>
<td>N71E</td>
<td>545 ± 33</td>
<td>123 ± 8</td>
<td>2.62 ± 0.43</td>
<td>0.5 x 10$^5$</td>
</tr>
<tr>
<td>V63FN71E</td>
<td>648 ± 40</td>
<td>146 ± 9</td>
<td>1.31 ± 0.27</td>
<td>1.1 x 10$^5$</td>
</tr>
</tbody>
</table>

Table 6.5: Kinetic results for the diC$_{24}$PC micellar assay. Assay conditions: 0.2 µg (15 pmol) of enzyme, 25 mM calcium chloride, 100 mM sodium chloride and 1 mM sodium tetraborate pH 8.0 at 45°C. The sodium hydroxide concentration used as the titrant was 10 mM.

It must be emphasised that the reduction in $k_{\text{cat}}$ could be due to the enzyme no longer "recognising" the lipid-water interface. Thus non-productive binding of the enzyme to micelles of diC$_{24}$PC is observed thus giving a much lower $k_{\text{cat}}$ value, rather than alterations at
the active site. The double mutant seems to have regained some ability to bind micelles as shown by its lower $K_m^{PP}$ compared to N71E, thus showing the importance of the Phe-63 substitution. It is probably more useful to compare the $k_{cat}/K_m^{PP}$ values which reflects the enzymes specificity for a given substrate. For the native enzymes the values are similar; V63F shows a significant increase reflecting its increased efficiency at the micelle interface. Both N71E and V63FN71E have reduced specificity for diC8PC micelles (by a factor of ~20 and ~9 respectively).

6.5 $k_{cat}$ determination using the diC8PC monomer assay.

In order to establish whether the catalytic site had been altered in some way by the mutations, the activity on phospholipid monomers was measured using the pH-stat. 1,2-dihexanoyl-sn-glycero-3-phosphocholine (diC8PC) has a high cmc (7.5 mM) and thus is useful for measuring the turnover of monomeric substrate, rather than at a micellar interface. The major problem is that PLA2 has a very low turnover rate on monomer substrate and this is difficult to measure using the pH-stat. Therefore higher protein concentrations had to be used in order to obtain measurable rates. Attempts were made to measure the affinity for monomer substrate (i.e. $K_m$), but it proved too difficult to obtain reproducible results. Thus only turnover rates at 5 mM substrate are shown in Table 6.6. It is possible that even at these high substrate concentrations some of the enzymes may still not have been saturated with ligand. Porcine PLA2 has the greatest activity of all the enzymes assayed, V63F was found to have activity that was intermediate to that of porcine and bovine PLA2. What is clear from the results in Table 6.6 is that the Glu-71 substitution in both the single and double mutants alters the rate of hydrolysis on monomer substrate. N71E and V63FN71E have reduced rates of hydrolysis by a factor of ~2.5 and ~1.7 (compared to bPLA2) respectively. It is not clear whether this is due to an increase in $K_m$ (hence the enzyme may not be saturated) or a decrease in $k_{cat}$. 
Chapter 6: Kinetic characterisation of bPLA$_2$ mutants.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity (µmoles H$^+$/min/mg)</th>
<th>Turnover rate ($s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{\alpha}$bPLA$_2$</td>
<td>3.45 ± 0.3</td>
<td>0.78 ± 0.10</td>
</tr>
<tr>
<td>$^{\alpha}$pPLA$_2$</td>
<td>6.75 ± 1.0</td>
<td>1.52 ± 0.20</td>
</tr>
<tr>
<td>$^{\alpha}$V63F</td>
<td>4.38 ± 0.4</td>
<td>0.98 ± 0.09</td>
</tr>
<tr>
<td>$^{\alpha}$N71E</td>
<td>1.3 ± 0.1</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>$^{\alpha}$V63FN71E</td>
<td>2.0 ± 0.1</td>
<td>0.44 ± 0.02</td>
</tr>
</tbody>
</table>

Table 6.6: Results of the activity of the various PLA$_2$s with 5 mM diC$_{14}$PC. Due to the low activity of all the enzymes on diC$_{14}$PC, high protein concentrations had to be used as indicated $^{\alpha}$20 µg (1.5 nmoles) or $^{\alpha}$40 µg (3 nmoles). Assay conditions: 25 mM calcium chloride, 100 mM sodium chloride and 1 mM sodium tetraborate pH 8.0 at 45°C. The sodium hydroxide concentration used as the titrant was 5 mM.

6.6 Kinetic characterisation with long-chain phospholipids.

A variety of long acyl chain (C$_{14}$) phospholipids with different head-groups were assayed using the pH-stat to investigate whether the mutations introduced into the surface loop region had changed the substrate preference. Fig. 6.6 shows the types of substrate used. The major drawback of using long chain phospholipids is that they prefer to form vesicles rather than micelles. Once solubilised (by heating and sonication) they form turbid solutions. PLA$_2$ does not hydrolyse zwitterionic substrates efficiently when in vesicle form and long lag phases are observed. Thus for diMPE and diMPC the substrate was dispersed to form mixed micelles using 15 mM deoxycholate. Deoxycholate had the added advantage of allowing the enzyme to have a higher affinity for the interface due to its negative charge, thus avoiding the lag phase.
Chapter 6: Kinetic characterisation of bPLA₂ mutants.

DiMPG and diMPM did not require the presence of the deoxycholate and were rapidly hydrolysed even in the form of vesicles. When using deoxycholate the concentration of calcium was reduced as higher concentrations (above or at 25 mM) caused precipitation and a large reduction in activity as the substrate/deoxycholate was removed from solution. Only specific activities were measured as the kinetic characteristics of PLA₂ on mixed micelles and vesicles are difficult to interpret.
Table 6.7: Kinetic results of various PLA$_2$s with mixed micelles of 5 mM diMPC/15 mM deoxycholate. Assay conditions: 1 µg enzyme (75 pmoles), 10 mM calcium chloride, 100 mM sodium chloride and 1 mM sodium tetraborate pH 8.0 at 45°C. The sodium hydroxide concentration used as the titrant was 5 mM.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity ($\mu$moles H$^\cdot$/min/mg)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>Relative to wild type bPLA$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>bPLA$_2$</td>
<td>114 ± 9</td>
<td>26 ± 2</td>
<td>1.0</td>
</tr>
<tr>
<td>pPLA$_2$</td>
<td>242 ± 20</td>
<td>54 ± 5</td>
<td>2.1</td>
</tr>
<tr>
<td>V63F</td>
<td>207 ± 5</td>
<td>47 ± 1</td>
<td>1.8</td>
</tr>
<tr>
<td>N71E</td>
<td>91 ± 2</td>
<td>20 ± 0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>V63FN71E</td>
<td>147 ± 6</td>
<td>33 ± 1</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Table 6.8: Kinetic results with mixed micelles of 5 mM diMPE/15 mM deoxycholate. Assay conditions: 2 µg enzyme (150 pmoles), 2.5 mM calcium chloride, 100 mM sodium chloride and 1 mM sodium tetraborate pH 8.0 at 45°C. The sodium hydroxide concentration used as the titrant was 5 mM.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity ($\mu$moles H$^\cdot$/min/mg)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>Relative to wild type bPLA$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>bPLA$_2$</td>
<td>67 ± 2</td>
<td>15 ± 0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>pPLA$_2$</td>
<td>51 ± 1</td>
<td>11 ± 0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>V63F</td>
<td>114 ± 1</td>
<td>26 ± 0.2</td>
<td>1.7</td>
</tr>
<tr>
<td>N71E</td>
<td>77 ± 6</td>
<td>17 ± 1.3</td>
<td>1.1</td>
</tr>
<tr>
<td>V63FN71E</td>
<td>80 ± 2</td>
<td>18 ± 0.45</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Tables 6.7 and 6.8 show the results obtained using neutral zwitterionic substrates in mixed micelles. Even with the lower calcium concentrations, a limited precipitation of the reaction mixture was observed. This was unavoidable as the enzymes have very little activity on the substrate in the absence of deoxycholate. All the enzymes prefer the phosphatidylcholine substrate rather than the ethanolamine which is reflected in the higher $k_{cat}$ values with this substrate. The $pK_a$ of ethanolamine is 9.5 (Dawson et al., 1986), and so at pH 8 the head group should be protonated. Porcine PLA$_2$ and V63F have similar activities on the diMPC, but the mutant bovine enzyme has a significantly higher turnover rate on diMPE mixed micelles. It appears that on both types of mixed micelles the N71E has similar kinetics as the native bovine enzyme and V63FN71E has a slightly higher activity than bPLA$_2$. It must
be remembered that the observed rates will have been lowered, since the substrate has been
diluted by a factor of four at the interface by the presence of 15 mM deoxycholate.

To investigate whether the binding of N71E and V63FN71E to mixed micelles was
due to the negative charge introduced by deoxycholate at the interface, assays were carried out
using 5 mM diC4PC in the presence and absence of deoxycholate (Table 6.9).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity (µmoles H⁺/min/mg)</th>
<th>± deoxycholate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No deoxycholate 15 mM deoxycholate</td>
<td></td>
</tr>
<tr>
<td>bPLA₂</td>
<td>1325 ± 66  350 ± 21</td>
<td>0.26</td>
</tr>
<tr>
<td>N71E</td>
<td>355 ± 28   305 ± 27</td>
<td>0.83</td>
</tr>
<tr>
<td>V63FN71E</td>
<td>383 ± 38   260 ± 21</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Table 6.9: Kinetic assay results with diC₄PC in the absence and presence of deoxycholate. Assay conditions: 0.2
µg enzyme (15 pmoles), 10 mM calcium chloride, 100 mM sodium chloride and 1 mM sodium tetraborate pH
8.0 at 45°C. The sodium hydroxide concentration used as the titrant was 5 mM.

In the absence of any deoxycholate, the mutants have ~30% of the enzyme activity of
bPLA₂, reflecting the lower affinity for the lipid-water interface. The addition of 15 mM
dehoxycholate caused a reduction in the observed activity of the native bovine enzyme, due to
the surface dilution of the substrate. Yet this is not seen to the same extent for the single or
double mutants. For both enzymes the enzyme activity is now similar to that of bPLA₂. This
suggests that the introduction of a net negative charge to the lipid-water interface can shift the
E =⇒ E* equilibrium (see Discussion section, Fig. 6.12). That is, in the presence of micelles
of pure diC₄PC the mutant enzymes favour the E form and thus have a lower affinity for the
lipid-water interface. On the addition of a negative charge to the micelle interface, the
equilibrium is now shifted to favour the E* form of the enzyme which has a higher affinity for
the lipid-water interface and so the enzyme may now be in the highly processive scooting
mode of catalysis.
Chapter 6: Kinetic characterisation of bPLA₂ mutants.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity (μmoles H⁺/min/mg)</th>
<th>k_{cat} (sec⁻¹)</th>
<th>Relative to wild type bPLA₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>bPLA₂</td>
<td>1167 ± 11</td>
<td>263 ± 2.5</td>
<td>1.0</td>
</tr>
<tr>
<td>pPLA₂</td>
<td>1505 ± 5</td>
<td>339 ± 1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>V63F</td>
<td>1664 ± 50</td>
<td>374 ± 11.0</td>
<td>1.4</td>
</tr>
<tr>
<td>N71E</td>
<td>1888 ± 4</td>
<td>425 ± 1.0</td>
<td>1.6</td>
</tr>
<tr>
<td>V63FN71E</td>
<td>1780 ± 20</td>
<td>401 ± 4.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 6.10: Kinetic results for various PLA₂s with diMPG vesicles. Assay conditions: 5 mM diMPG, 0.25 μg enzyme (18.75 pmoles), 10 mM calcium chloride, 100 mM sodium chloride and 1 mM sodium tetraborate pH 8.0 at 45°C. The sodium hydroxide concentration used as the titrant was 5 mM.

Table 6.11: Kinetic results of PLA₂ assays using diMPM vesicles. Assay conditions: 5 mM diMPM, 0.25 μg enzyme (18.75 pmoles), 10 mM calcium chloride, 100 mM sodium chloride and 1 mM sodium tetraborate pH 8.0 at 45°C. The sodium hydroxide concentration used as the titrant was 5 mM.

Tables 6.10 and 6.11 show the results obtained using the negatively charged phospholipids diMPG and diMPM in the absence of deoxycholate. DiMPG and diMPM do not form micelles due to their long alkyl chains, but probably form larger aggregated structures such as vesicles. On diMPG a short lag phase was observed (10-15 seconds), after which the rate was linear for several minutes. All the enzymes have high turnover rates and all the mutants have activities marginally greater than native bovine and porcine PLA₂.

DiMPM was used since PLA₂ has a high affinity for the negatively charged interface. The binding is so tight (K_d ~0.1 pM [Jain and Berg, 1989]) that the enzyme does not leave the interface of the vesicle (i.e. the E* form of the enzyme is favoured in the E ⇌ E* equilibrium at the lipid-water interface). When using diMPM, the assay conditions were such that vesicle fusion was occurring (Berg et al., 1991) due to the addition of the stock calcium
solution. The results are similar to those measured using diMPG, except that greater rates were observed on the diMPM vesicles. The rates measured using both substrates with all the enzymes are in general very similar.

6.7 Kinetic characterisation using a spectrophotometric assay.

2-hexadecanoylthio-1-ethylphosphorylcholine (HEPC) was used to measure the activity of the mutant proteins via a spectrophotometric assay (Bhat et al., 1993a). HEPC is a thio-lysophospholipid in which the oxygen ester bond has been replaced by a thio ester bond. The overall assay reactions are shown in Fig. 6.7 described below.

Fig. 6.7: Schematic representation of the DTNB/HEPC spectrophotometric assay (modified from Balet et al., 1988).
Chapter 6: Kinetic characterisation of bPLA₂ mutants.

Hydrolysis of HEPC (1) by PLA₂ produces a fatty acid (2) and a free sulphhydryl group (3) which is readily trapped by 5,5-dithiobis (2-nitrobenzoic acid) (DTNB, (4)), to produce a mixed disulphide (5) and a highly absorbing thiolate (6). Thus the rate of hydrolysis of HEPC can be measured by monitoring the thiolate (6) production by following the absorbance at 405 nm. The amount of product produced was determined from a calibration curve using 2-mercaptoethanol (1-20 nmol) as the free thiol agent.

The main reason for using this assay was that it was hoped it would allow kinetic parameters on both monomer and micellar substrates to be measured, since the cmc was stated to be 0.5 mM (Bhat et al., 1993a). However, the cmc determined here using the ANS fluorescence method gave a value of 5 μM (see Table 6.1), two orders of magnitude lower than the published value (Bhat et al., 1993a). If the cmc was 0.5 mM then an increase in enzyme activity should have been observed when passing from the monomer to the micellar state. This activation however was not observed and so the assay could only be used to determine kinetic constants on micellar substrate. The assays were carried out in a microtitreplate using a microtitreplate reader to measure the absorbance during the course of the assay. The reproducibility of the assay was low and this was attributed to the precipitation of the product of the hydrolysis, hexadecanoic acid (Fig. 6.7 (2)). Hexadecanoic acid is insoluble in aqueous solutions above ~10 μM and has a melting point of ~60°C. Thus, the product formed small aggregates which scattered the light during the assay and produced elevated absorbance readings. Fig. 6.8 shows a typical rate curve of HEPC with bPLA₂. The effect of the light scattering on the measured A₄0₅ only appears to have a significant contribution after approximately 15 minutes. Thus in order to avoid the effect of light scattering on observed enzymatic rate, the initial rate was only measured during the first 15 minutes of the assay. An example of the fitted data is shown in Fig. 6.9. Kinetic parameters for HEPC, Kₘₚₚ and kᵦᵦ are shown in Tables 6.12 and 6.13.
Chapter 6: Kinetic characterisation of bPLA2 mutants.

Fig. 6.8: The $A_{405}$ change observed (solid line) during the hydrolysis of 2 mM HEPC with 0.8 µg of bPLA2 at 37°C. The dashed line represents the initial rate as determined over the first 15 minutes.

Fig. 6.9: Computer fitted data for the initial rates of hydrolysis of HEPC by N71E. Assay conditions: 5 µg (375 pmoles) enzyme, 50 mM sodium chloride, 50 mM calcium chloride, 200 mM Tris-HCl pH 8.0 at 37°C. The $K_m$ and $V_{max}$ are shown.
Chapter 6: Kinetic characterisation of bPLA₂ mutants.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m^{app}$ (µM)</th>
<th>Relative to wild type bPLA₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>bPLA₂ (1 µg)</td>
<td>796 ± 83</td>
<td>1</td>
</tr>
<tr>
<td>pPLA₂ (0.25 µg)</td>
<td>5.2 ± 1</td>
<td>6.8 x 10⁻³</td>
</tr>
<tr>
<td>V63F (0.5 µg)</td>
<td>7.1 ± 1</td>
<td>9.2 x 10⁻³</td>
</tr>
<tr>
<td>N71E (5 µg)</td>
<td>4494 ± 450</td>
<td>5.8</td>
</tr>
<tr>
<td>V63FN71E (1 µg)</td>
<td>3145 ± 310</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Table 6.12: Kinetic results for HEPC and PLA₂. Assay conditions as for Fig. 6.9, the various amounts of enzyme used in the assay are shown in brackets.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>Relative to wild type bPLA₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>†bPLA₂ (1 µg)</td>
<td>18.4 ± 0.03</td>
<td>1</td>
</tr>
<tr>
<td>‡pPLA₂ (0.25 µg)</td>
<td>26.2 ± 0.03</td>
<td>1.4</td>
</tr>
<tr>
<td>†V63F (0.5 µg)</td>
<td>13 ± 0.001</td>
<td>0.71</td>
</tr>
<tr>
<td>*N71E (5 µg)</td>
<td>5.3 ± 0.3</td>
<td>0.29</td>
</tr>
<tr>
<td>*V63FN71E (1 µg)</td>
<td>10.1 ± 0.5</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Table 6.13: $k_{cat}$ values for various PLA₂s on HEPC micelles. The HEPC concentrations were 15 ± 1 mM. * The $k_{cat}$ values for N71E and V63FN71E were determined from the calculated from the computer fitted data for the $K_m^{app}$.

Porcine PLA₂ has ~150 fold higher affinity for the interface compared to bPLA₂. The V63F mutant again shows that its binding is characteristically more similar to porcine than bovine PLA₂. The N71E and V63FN71E mutants have a significantly reduced ability to bind to the interface, although the presence of the Phe-63 in the double mutant does seem to enhance the binding slightly. The $k_{cat}$ values obtained with this substrate also reflects the trends seen in the other kinetic assays. What is apparent from the results shown in Table 6.13 is that all the enzymes show a much lower turnover rate on the thio-ester bond compared to the oxygen ester bond found in phospholipids. The porcine enzyme has a slightly greater activity than the native bovine enzyme, the V63F mutant again displays turnover rates similar to bovine PLA₂. The N71E and V63FN71E mutants have a much reduced activity on HEPC, the largest difference is seen in N71E whose activity is less than a third of that of the bovine
protein; the double mutant has half the activity of native enzyme. The concentration of HEPC at which the $k_{cat}$ values for all the proteins (except for N71E and V63FN71E) were obtained were well above the $K_m$; the enzymes were therefore probably saturated with substrate. For the N71E and V63FN71E, the $k_{cat}$ were calculated from the $V_{max}$ determined from the computer fit of the $K_m$ data, measured over a range of non-saturating substrate concentrations.
6.8 HEPC spectrophotometric microtitre-plate assay.

Although PLA$_2$ specifically hydrolyses the ester linkage at the sn-2 position of phospholipids, this is not the minimal substrate requirement. It has been shown that the minimal substrate requirement of the enzyme is an acyl ester which has to be present in a position adjacent to the C-O-P linkage and an acyl group which has to occupy a certain stereochemical configuration (de Haas and van Deenen, 1963; Fig. 6.10). It has been shown that phosphononolecithin can also be degraded by PLA$_2$, but at a very low rate (Kuipers et al., 1990).

![Fig. 6.10: The minimal substrate requirements for PLA$_2$. Taken from (a) de Haas and van Deenen, 1963 and (b) Kuipers et al., 1990.](image)

Hence, even monoacyl chain ligands can be hydrolysed if they have the minimum substrate requirement. The acyloxy ester bond can also be replaced by an acylthio ester bond which still allows PLA$_2$ to turnover the substrate. The major drawback is that these analogues possess only weak substrate properties. The oxyester detergents have weaker substrate characteristics than the thioester ligands (Aarsman and van Den Bosch, 1979). The increase in $k_{cat}$ upon O$\rightarrow$S substitution has been attributed to the better leaving properties of the SH-containing product, assuming that the decomposition of the tetrahedral intermediate is the rate limiting step (Aarsman and van Den Bosch, 1979; van Oort et al., 1985a). The highest
Chapter 6: Kinetic characterisation of bPLA₂ mutants.

measured PLA₂ activity was for n-heptanoylthioglycol sulphate and gave a specific activity of 20 μmoles/min/mg with porcine PLA₂ (van Oort et al., 1985b). The substitution of O→S also reduces the cmc of the substrate when compared to the identical substrate containing the oxygen ester bond. This is due to the increased hydrophobic nature of the sulphur atom (van Oort et al., 1985a; Aarsman and van Den Bosch, 1979).

HEPC was first synthesised by Aarsman et al., (Aarsman et al., 1976) but a more simplified synthesis method was shown by Bhat et al., (Bhat et al., 1993a). The HEPC spectrophotometric assay was used for several reasons. Firstly, spectrophotometric assays are more sensitive than the titrametric pH-stat assay, this would allow the accurate measurement of low enzymatic rates for the mutant proteins described in this work. Due to the low volumes required for the assay (200 μL) only small quantities of substrate are needed. Using the microtitre plate also allows the rapid assaying of a large number of samples in a short period of time. The most compelling reason for using HEPC was as a monomolecular assay as the cmc of the ligand was quoted as being between 0.3-0.5 mM (Bhat et al., 1993a; 1993b). The turnover rates of monomer substrates are very low (approximately 1 s⁻¹ for native bovine PLA₂) as has been shown on diC₆PC and this is close to the detection limit of the pH-stat. Thus a spectrophotometric assay would allow the accurate measurements of the kinetic parameters (kcat and Km) on monomolecular substrate through the cmc to micellar substrate aggregates.

Although HEPC is hydrolysed by PLA₂, the observed enzymatic rates are very low, for example the highest measured rate was for pPLA₂ with HEPC was 2 μmoles/min/mg (kcat= 0.4 s⁻¹) which is five fold lower than for n-octanoylthio-1-ethylphosphophorylcholine (van Oort et al., 1985a). This is probably due to the long C₁₆ acyl chain of HEPC which may have problems fitting into the active site of the enzyme. The low rates are still well within the detection level of the assay as turnover rates as low as 0.04 s⁻¹ were able to be measured for the N71E mutant.

Initial experiments using HEPC failed to show the expected increase in activity in PLA₂ on passing the reported cmc of 0.5 mM (Bhat et al., 1993a). It was suspected that the cmc of HEPC was appreciably lower than 0.5 mM as HEPC has a similar structure to C₁₆PN (cmc = 5 μM), and so the cmc was measured using the ANS fluorescence method (de
Chapter 6: Kinetic characterisation of bPLA$_2$ mutants.

Vendittis et al., 1981). The cmc was found to be 5 μM (Table 6.1), which is over two orders of magnitude lower than that measured by Bhat et al. The cmc of HEPC has been measured previously (Aarsman and van Den Bosch, 1979) and found to be between 3-5μM. Thus HEPC can not be used as a monomer assay for PLA$_2$ and only as a micellar assay. This limits its usefulness as the combination of long acyl chain and zwitterionic head group significantly diminish the substrate quality of the ligand (van Oort et al., 1985a).

The $K_m^{app}$ of porcine PLA$_2$ for HEPC micelles was found to be 5.7 μM, which is similar to the value obtained by Aarsman et al., of 10 μM (Aarsman and van Den Bosch, 1979).

6.9 V63F bPLA$_2$.

The binding experiment measurements for V63F are summarised in Fig. 6.11.

![Figure 6.11](image.png)

Fig. 6.11: Summary of the affinity of V63F and authentic PLA$_2$s for a variety of interfaces. The bars represent $\square$ bPLA$_2$, $\Box$ pPLA$_2$, and $\blacksquare$ V63F. Note the logarithmic scale.

Comparing the results from different assays is not possible, as the packing interactions between phospholipids vary between micelles, vesicles and mixed micelles which thus effects the kinetic properties of the enzyme at the different lipid-water interfaces. However, direct and detailed comparison of bPLA$_2$ and the mutants for each assay is acceptable.
Chapter 6: Kinetic characterisation of bPLA$_2$ mutants.

What is clear from Fig. 6.11 is that the Val-63→Phe substitution in bPLA$_2$ alters the binding characteristics of the mutant so that is more similar to pPLA$_2$. Fig. 6.12 schematically represents the mechanism of interfacial activation and for the purpose of this discussion only the E ↔ E$^*$ equilibrium will be considered. The E ↔ E$^*$ equilibrium represents the binding step of the enzyme to the lipid-water interface. The E form of the enzyme free in solution has low activity, but on binding to a lipid-water interface the protein undergoes a structural change to give the catalytically active E$^*$ species. The non-hydrolysable lysophospholipid analogue C$_{16}$PN was used to provide a model lipid-water interface so that the binding affinity of the various enzymes could be compared.

Fig. 6.12: The proposed kinetic model of interfacial activation of PLA$_2$ on aggregated substrates (modified from Jain and Berg, 1989). According to the above scheme, the enzyme free in solution (E) undergoes a structural change on binding to the lipid-water interface and becomes activated (E$^*$). Once at the interface the enzyme binds a substrate molecule (S) to form the enzyme-substrate complex (E$^*$S). The substrate is hydrolysed and the product (P) is formed, after which the enzyme is free to hydrolyse further phospholipid molecules or (depending on the affinity for the lipid-water interface) leave the micelle/vesicle (see Chapter 1, Section 1.8).

The V63F mutation has shifted the E ↔ E$^*$ equilibrium further to the right compared to authentic bPLA$_2$, so that in the presence of zwitterionic micelles the E$^*$ form of the enzyme is favoured. This shift is reflected in the lower $N$K$_d$ value of V63F (as compared to authentic bPLA$_2$) for micelles of C$_{16}$PN. The $N$K$_d$ represents the value where 50% of the enzyme is
Chapter 6: Kinetic characterisation of bPLA$_2$ mutants.

saturated by micelles, $N$ being the number of monomers in the micelle and $K_d$ the dissociation constant. Thus low $N$-$K_d$ values reflect a high affinity of the enzyme for the micellar interface. The $N$-$K_d$ values for the native proteins increased slightly as the pH was increased from pH 5 to 8, which might be expected due to the basic nature of these enzymes. The mutants binding increases by almost five-fold from $N$-$K_d = 0.42$ to $0.093$ mM on increasing the pH to 8. These results suggest that there may be an ionisable group involved in the binding of the enzyme to the detergent-water interface.

In general the kinetic characteristics of V63F are more similar to the bovine enzyme than pPLA$_2$. V63F does display greater activity on a wide range of substrates but there has been a clear improvement on neutral zwitterionic phospholipids (Fig. 6.13). The turnover on monomeric substrate is slightly greater than the native enzyme and this may account for the approximately 30% increase in activity on aggregated substrate.

The greatest difference between the native enzymes is on the egg lecithin assay, which shows that the porcine enzyme has a five-fold increase in the turnover rate compared to the bovine enzyme. Clearly the V63F mutation in bPLA$_2$ does not impart the kinetic characteristics of the porcine enzyme. The mutant does however show a clear preference for zwitterionic phospholipids: the $k_{cat}/K_m$ for the V63F mutant is ~3 fold higher than that of the native bovine enzyme ($24 \times 10^3$ M$^{-1}$s$^{-1}$ and $9.8 \times 10^3$ M$^{-1}$s$^{-1}$ respectively) with diC$_2$PC (Fig. 6.14). This increase reflects the improved properties of the enzyme, primarily through a reduction in $K_m$. 
Chapter 6: Kinetic characterisation of bPLA$_2$ mutants.

![Graphs and charts]

Fig. 6.13: Turnover rates (s$^{-1}$) of authentic PLA$_2$S and V63F on a variety of phospholipids. The bars represent bPLA$_2$, pPLA$_2$ and V63F.

Fig. 6.14: A comparison of the specificity constants of authentic PLA$_2$S and V63F with diC$_8$PC micelles.
To analyse how the V63F mutation may have caused the observed change in interfacial binding, previously published X-ray crystal structures and nmr solution structures of several pancreatic PLA$_2$ were compared. Regions of low electron density occur in the density maps of the crystal structures indicating that the conformation of the backbone and side chains are difficult to determine. This is reflected in the temperature factor or B-factor. The B-factor reflects the thermal motion or the occupancy of multiple sites by the side chain, thus the greater the value of the B-factor the greater the disorder. An indication of relative disorder for a side chain can be inferred by a comparison of the B-factors of a residue involved in stable secondary structures (such as an $\alpha$-helix), and a surface exposed side chain. So it must be emphasised that some of the regions of the crystal structures that are poorly defined, and this may be due to mobile regions of the secondary structure. In PLA$_2$ the regions with the largest B-factors are the N-terminus, the 58-71 surface loop, the Ca$^{2+}$ binding loop, the C-terminus and also loop regions which join the secondary structural elements (Dijkstra et al., 1981a; 1983). These mobile regions are also evident from the nmr solution structure of pPLA$_2$ (see Fig. 6.15).
Chapter 6: Kinetic characterisation of hPLA₂ mutants.

Fig. 6.15: The 20 conformers of porcine pancreatic PLA₂ free in solution as determined by nmr (left), and average nmr structure with Phe-63 displayed (right) (van den Berg et al., 1995b).

This is the only nmr solution structure of a PLA₂ available at this time (van den Berg et al., 1995a; 1995b). The average nmr structure (determined from ~20 calculated structures) does appear to have the general conformation as the crystal structure (Finzel et al., 1991; Dijkstra et al., 1983). The structures of the enzyme free in solution and in the ternary complex were solved. The ternary complex consists of the enzyme bound to a micelle of dodecylphosphocholine (DPC) with a strong competitive inhibitor ((R)-1-thio-octyl-2-heptylphosphonyl-1-deoxyglycerol-3-phospha) bound to the active site (van den Berg et al., 1995a; 1995c). There are some significant differences between the enzyme free in solution and in the ternary complex. When free in solution the regions with the greatest disorder are the Ca²⁺ binding region, residues 1-3 of the N-terminus, the C-terminus and the 58-72 surface loop (van den Berg et al., 1995a; 1995b). These regions are ill-defined as the correlations for a number of backbone resonances were weak or missing (van den Berg et al., 1995b). The N-terminus of the protein free in solution is evidently not hydrogen bonded to the active site as determined by nmr, as no correlations were observed for Ala-1. Thus the active site residues
Chapter 6: Kinetic characterisation of bPLA2 mutants.

are mobile and the first three residues of the N-terminus are disordered (Peters et al., 1992). An ordered N-terminal helix and active site only appear to be complete in the ternary complex. Thus the authors have concluded that this may explain why the enzyme has low activity on monomer substrates, as the vital active site catalytic network has not fully formed. Only when the enzyme is at the interface is the network complete, with the active site residues in their catalytically optimal conformation and thus an increase in the activity of the enzyme is observed. The ordering of the N-terminus has also been observed on binding the enzyme to micelles of the detergent sodium dodecylsulphate (Kilby et al., 1995). The surface loop 58-71 is also ill-defined in the nmr solution structure (Fig. 6.15). The stabilisation of both the N-terminus and the surface loop is thought to be involved in the formation of the interfacial recognition site (IRS), which is the region of the enzyme responsible for recognising and binding to the lipid water interface. The stabilisation of the N-terminal helix and surface loop is thought only to occur once the enzyme is bound to a micelle with the active site occupied, as seen in the ternary complex (van den Berg, 1995a; 1995c).

The ternary nmr structure was found to resemble the crystal structure of free enzyme. The crystal structures of the enzyme alone are very similar to the structure determined with inhibitors bound at the active site (Scott et al., 1991). Thus there appears to be no structural change in the protein on binding ligand. It has been suggested that the conformation of the enzyme as observed in the crystal structure could be the result of a preferential crystallisation of one protein conformer, resembling the nmr solution structure of the enzyme bound to a lipid aggregate (van den Berg et al., 1995a; 1995b). Fig. 6.16 shows the high resolution nmr structure of pPLA2 free in solution overlaid with the crystal structure. What is evident is the differences at the N-terminus (which are of the order of ~8 Å), the Ca2+ binding loop and several surface loops. So there does appear to be significant differences between the nmr structure of the enzyme free in solution, and the X-ray determined crystal structure.
Thus it may be justified to assume that the X-ray determined crystal structures do represent the protein conformation, but at the micelle-water interface. It should also be realised that the packing interactions between the molecules in the crystal structures can influence the order of the surface side chains as they interact with neighbouring molecules. Fig. 6.17 shows the 58-71 surface loop region of the native enzymes (Dijkstra et al., 1983) and the porcine F63V mutant (Thunnissen et al., 1993).
Fig. 6.17: X-ray crystal structures of native bovine (2.6 Å resolution), porcine (3.0 Å resolution) (Dijkstra et al., 1983) and porcine F63V (2.2 Å resolution) PLA2 (Thunnessen et al., 1995). The residues shown are Val-63 (grey), Leu-64 (white), Val-65 (green), Asp-66 (purple) and Glu-71 (red).
Chapter 6: Kinetic characterisation of bPLA₂ mutants.

From the crystal structures it is clear that in general the tertiary structures are very similar and that the greatest difference is in the 58-71 surface loop. From the results discussed previously, it is clear that the V63F mutation has a significant effect on the binding properties of bPLA₂, to the extent that the mutant enzyme’s affinity for micelles is similar to that of pPLA₂. By comparing the crystal structures, a mechanism to explain the binding properties of V63F may be proposed.

In bPLA₂, the Val-63 residue is present on the surface of the α-helix pointing into solution, whereas Phe-63 in pPLA₂ is buried into a hydrophobic pocket. The nmr solution structure also shows that Phe-63 is directed inwards (Fig. 6.16). The F63V mutation has been shown to have altered the localised secondary structure, so that again the Val-63 is pointing into solution. The possibility that the conformation of the loop in F63V was due to interactions between closely packed molecules in the crystal was discounted because of the following observation. Wild-type bPLA₂ has been crystallised in two different space groups, but the three dimensional structures obtained from the two space groups were very similar. The 58-71 loop has the same general conformation in the two structures, although they make completely different contacts in the two crystal forms. The crystal contacts for F63V were different to those observed in the bovine structures, but the conformation of residues 58-64 did resemble the wild type bovine enzyme (Thunnissen et al., 1993).

A possible explanation for the increased binding of the V63F bovine mutant to micelles may be due to a localised change in the structure of the surface loop so that the Phe side chain now orientates itself into the hydrophobic pocket. In doing so the αC-backbone may be distorted forcing residues 64-66 towards the micelle binding region of the enzyme (as seen in the porcine enzyme), when the enzyme is at the micellar interface. As a result of this the hydrophobic side chains of residues Leu-64 and Val-65 would be closer to the interfacial recognition site (IRS) of the enzyme. Residues 64 and 65 have been implicated as part of the IRS (Pieterson et al., 1974; Dijkstra et al., 1984; 1981a, 1981b; Scott and Sigler, 1994). If they are closer to the enzyme-lipid interface they could make a greater contribution to micellar binding via hydrophobic interactions. The positions of Leu-64 and Val-65 are shown in Fig. 6.17. They are significantly closer to the interface in the porcine enzyme than in the bovine protein, the difference in the αC positions between the two proteins is ~8 Å for both amino
In the F63V mutant the change in the surface loop conformation due to Val-63 causes residues 64 and 65 to move away from the interface to a position which resembles that of bPLA₂. Yet the binding properties on C₁₄PN micelles of the F63V mutant do not change significantly ($N-K_d$ values are pPLA₂ = 122 μM and F63V = 456 μM) when compared to the large differences in binding between bovine PLA₂ and its V63F mutant described herein (Fig. 6.11). This may be because the remaining 18 amino acid residues (which differ in the primary amino acid sequence between the two enzymes) contribute significantly to micellar binding to such an extent that the effect of the F63V mutation cannot be precisely assessed. For example the residues at positions 6 and 17 in pPLA₂ are more basic (Arg-6 and His-17) than those in the bovine enzyme (Asn-6 and Glu-17). Thus on the basis of electrostatic interactions, pPLA₂ would display tighter binding towards negatively charged micelles. This is supported by an experiment in which a semisynthetic bPLA₂, which had the Asn-6 substituted by an Arg residue, displayed an affinity for aggregated substrates comparable to that of the porcine enzyme (van Scharrenburg et al., 1981). The contribution of hydrophobic interactions to micellar binding has also been investigated. Lugtigheid et al., observed a 60-fold decrease in the $K_m^{upp}$ of pPLA₂ for micellar substrates (diC₃PC) after acylation (C₁₄ chain length) of Lys-56 (Lugtigheid et al., 1993). Work carried out by van der Wiele et al. showed that the attachment of a long-chain acyl group to Lys-116 of pPLA₂ enhanced the binding to micellar interfaces (cis-9-octadecenylphosphocholine) and the ability to penetrate densely packed monolayer structures (van der Wiele et al., 1988a; van der Wiele et al., 1988b).

To investigate the difference in the binding properties between porcine and bovine PLA₂, it would be easier to interpret the effect of the amino acid substitutions in going from bovine→porcine rather than porcine→bovine, which is further complicated by the differences at the other 18 amino acid residues. The results for the V63F mutant of bPLA₂ described in this work show that Phe-63 is involved in increasing the affinity of the enzyme for the lipid-water interface and therefore Phe-63 may be responsible for the observed binding difference between the bovine and porcine enzymes. The enzymatic activity of V63F is more similar to the bovine enzyme than the porcine enzyme, although it does show an increase in activity of ~30% on a variety of phospholipids. This slight increase may be the result of a conformational...
change at the active site of the protein caused by a change in the conformation of the surface loop. Residues in this loop region contribute to interactions with the substrate, for example Tyr-69 which is involved in the binding of the phosphate moiety of phospholipids (Kuipers et al., 1989).

6.10 N71E bovine PLA₂.

In the otherwise highly conserved surface loop 58-71, the second residue difference between bPLA₂ and pPLA₂ is at position 71. In bPLA₂ an Asn residue is present at position 71 whereas a Glu is found in the porcine enzyme. Asn-71 in the bovine enzyme is also involved in several hydrogen bond interactions with Asp-66, Tyr-73 and Ala-1. The hydrogen bond between Asp-66 and Asn-71 is absent from the porcine enzyme presumably due to the charge-charge repulsion between these two side chains.

Fig. 6.18 summarises the results of the binding experiments involving N71E.

![Graph showing the affinity of authentic PLa₅s and N71E for a variety of interfaces.](image)

**Fig. 6.18:** Summary of the affinity of authentic PLA₂s and N71E for a variety of interfaces. The bars represent □bPLA₂, □pPLA₂ and ■N71E.

The data obtained from the C₁₆PN, diC₈PC and HEPC assays all show that N71E has a very low affinity for neutral zwitterionic interfaces. Even at the pH 5, the protein has a
significantly reduced affinity for \text{C}_{16}\text{PN} micelles when compared to native bPLA\textsubscript{2}. Therefore the E form of the enzyme predominates in the \( E \rightleftharpoons E^* \) equilibrium, even at low pH values. The results obtained from the zwitterionic mixed micelle assays (Fig. 6.19) seem to contradict the above observation, on the whole N71E appears to have activity similar to the native protein, and with negatively charged substrates (i.e. diMPG and diMPM) greater turnover rates are observed.

\begin{align*}
\text{Fig. 6.19: The turnover rates} & \left( \text{s}^{-1} \right) \text{of authentic PLA}_2 \text{s and N71E on a variety of phospholipids. The bars represent } & \square \text{bPLA}_2, \quad \square \text{pPLA}_2 \text{ and } \square \text{N71E.}
\end{align*}

The reason for the apparent inconsistency is two fold and can be seen with the results of the following experiment involving 5 mM diC\textsubscript{8}PC and the rates of hydrolysis measured in the absence and presence of 15 mM deoxycholate (Fig. 6.20). The kinetic constant \( K_m^{\text{app}} \)}
cannot be determined with mixed micelles as the enzyme does not follow Michaelis-Menten kinetics with the substrate aggregate and so only specific activities can be determined.

![Specific activity (µmoles/min/mg)](image)

**Fig. 6.20:** The specific activity of native bPLA$_2$, N71E and V63FN71E with 5 mM diC$_8$PC in the presence and absence of deoxycholate.

In the absence of detergent, the mutants showed a ~3-4 fold lower turnover rate compared to bPLA$_2$. This may be due to either the enzymes' activity being reduced by the presence of the mutation or a reduction in their ability to bind to the lipid-water interface. The presence of 15 mM deoxycholate reduces the catalytic rate of the bovine enzyme by a factor of ~4 due to surface dilution of the phospholipid by the detergent. The rate of hydrolysis on diC$_8$PC/deoxycholate micelles with both mutants is only slightly reduced compared to that in the absence of detergent. It therefore appears that in the presence of deoxycholate, all the enzymes have very similar turnover rates, whereas the actual situation is that the detergent reduces the observed turnover rate of the native enzyme due to surface dilution of the phospholipid. However, deoxycholate enhances the affinity of the mutant PLA$_2$s for the lipid-water interface by introducing a net negative charge at the interface where the enzymes now can hydrolyse the phospholipid at rates similar to the native enzyme. Therefore the rates measured using mixed micelles are under conditions where the mutant enzymes have a high affinity for the detergent-water interface. Under these conditions the N71E mutant actually has activity similar to the native bPLA$_2$ as a greater proportion of the enzyme is bound to the
mixed micelle than would be in the absence of the negatively charged detergent. Therefore in the E ↔ E* equilibrium, the E form of the mutant enzymes are favoured in the presence of pure micelles of diC_{3}PC, whereas with bPLA_{2} a greater proportion of the enzyme is in the active E* form. But with mixed micelles, where a negative charge has been introduced at the interface, the equilibrium of the mutant enzymes shifts to favour the E* species which now show activity similar to bPLA_{2}.

On diC_{3}PC, which under the assay conditions forms a monodispersed solution, N71E shows a significant decrease in its ability to hydrolyse the monomolecular substrate. Therefore the mutation must affect the catalytic site of the enzyme, and this is also reflected in the \( k_{\text{cat}}/K_{\text{m}} \) value of diC_{3}PC. The specificity constant has been reduced by a factor of 21 as compared to the native bovine enzyme due to a significant reduction in both the turnover rate and binding affinity for diC_{3}PC micelles (Fig. 6.14).

N71E shows characteristics on monomolecular and micellar substrates which are strikingly similar to proPLA_{2}. ProPLA_{2} (which will be referred to as the zymogen) is the inactive form of PLA_{2}, which is secreted from the pancreas. The zymogen is expressed with a heptapeptide at its N-terminus, the removal of which by trypsin produces the activated form of the enzyme (de Haas et al., 1968; Abita et al., 1972). The presence of the N-terminal extension prevents the formation of the hydrogen bonding network between Ala-1 and the active site, thus significantly reducing the activity of the zymogen. As with N71E, the zymogen hydrolyses monomer substrate at a lower rate than the active form of the enzyme, for example the \( V_{\text{max}} \) for the porcine zymogen is reduced by ~50% compared to native enzyme on monodispersed diC_{3}PC (Pieterson et al., 1974). The ability of the zymogen to recognise organised lipid-water interfaces is virtually non-existent, and it shows no increase in activity as the substrate concentration increases past the cmc of the phospholipid. The affinity of the zymogen for micelles of C_{16}PN is significantly increased on the addition of negatively charged detergents to the micelle (Volwerk et al., 1984). Thus the kinetic behaviour of the zymogen and the information gained from the X-ray crystal structures may help to determine the nature of the effect of the N71E substitution.

The kinetic characteristics of the zymogen have been attributed to the structural differences between the inactive and active form of PLA_{2} (Dijkstra et al., 1982). Inspection of
the crystal structure of the zymogen shows that there are several regions of the enzyme which are ill-defined including the N-terminus and the surface loop region 58-71 (Dijkstra et al., 1982). In the crystal and nmr solution structure of the mature enzyme these regions are also disordered, but on micellar binding the $\alpha$-NH$_3^+$ of Ala-1 interacts with the active site so that the 58-71 surface loop and N-terminal helix adopt a more rigid conformation, and this allows the formation of the IRS. Thus it has been reasoned that the inability of the zymogen to recognise organised lipid-water interfaces is because Ala-1 fails to form the interaction with the active site due to the prosequence (Dijkstra et al., 1984), the 58-71 surface loop/N-terminal helix remain mobile and disordered, and thus the IRS fails to form. So a similar effect in which the surface 58-71 loop remains disordered and the IRS fails to form, may be occurring with N71E. The crystal structures of the native bovine and porcine enzyme are shown in Fig. 6.21.

Fig. 6.21: The localised environment around residue 71 of bovine (left) and porcine (right) PLA$_2$. The hydrogen bond distances shown are in Å.

What is clear from Fig. 6.21 is that the substitution of Asn-71 for Glu will cause serious problems with side chain packing in the mutant. The Glu side chain is longer by a methylene moiety and is negatively charged compared to Asn. In pPLA$_2$, Glu-71 is
accommodated by the conformation of the 58-71 surface loop. Glu-71 causes a significant change in the loop conformation so that Asp-66 points out into solution. In the F63V porcine mutant (see Fig. 6.17), Asp-66 shows a significant shift to a position which is similar to Asp-66 of the bovine enzyme, but not identical, as an electrostatic clash between Asp-66 and Glu-71 would occur. Thus to accommodate Glu-71 into the N71E structure, a local conformational change may occur, so that Asp-66 may now point away from 71 and into solution. Another feature of Asn-71 in the bovine protein is the hydrogen bonding system involving this residue. With the Glu-71 mutation, the interactions with Tyr-73 and Asp-66 may be lost and this may prevent the formation of the IRS on micellar binding as the surface loop remains disordered. From the measured B-factors it is clear that in bPLA$_2$ the Asn-71 side chain is constrained (Dijkstra et al., 1981a) and may be due to its involvement with Tyr-73 and Asp-66. It could also be possible that in order to accommodate the Glu-71 side chain in the mutant, the hydrogen bond between Ala-1 and the backbone C=O of 71 may be lost due to an increased distance between the atoms. Alternatively, the hydrogen bond between Ala-1 and the backbone C=O of 71 could be retained, but in order to accommodate Glu-71, Ala-1 may have moved thus disrupting other hydrogen bonds. This would explain the reduction in the rates observed with monomolecular substrates and N71E. It has previously been demonstrated that any disruption of the N-terminal $\alpha$-NH$_2$* interaction (for example by chemical modification or the presence of a pro-sequence) with the active site changes the kinetic characteristics of the modified enzyme to that of the zymogen (Verheij et al., 1981; Dijkstra et al., 1984). The transamination of the N-terminus produced a modified enzyme with zymogen like properties, and this was also seen from the crystal structure of the transaminated protein. Again, regions of electron density were missing for the N-terminus and the surface loop region 58-71. Thus it was concluded that the loss of the N-terminal $\alpha$-NH$_3$*/active site hydrogen bonding network prevented the formation of the IRS by the stabilisation of the surface loop and N-terminal $\alpha$-helix and hence the enzyme lost its ability to recognise organised lipid-water interfaces (Verheij et al., 1981). At the present time there is no structural information available on N71E and therefore the structural consequences of the mutation cannot be assessed.

Alternatively the decreased affinity of the N71E mutant for neutral phospholipid-water interfaces may simply be due to the presence of the negative charge at the IRS of the enzyme.

Chapter 6: Kinetic characterisation of bPLA$_2$ mutants.
Chapter 6: Kinetic characterisation of bPLA2 mutants.

This may have been predicted by the complementary charge model of enzyme binding to the lipid-water interface, i.e. introducing a positive charge to the IRS would enhance binding to the lipid-water interface, whereas the addition of a negative charge would decrease the affinity of PLA2 for the interface. It has been shown previously that the substitution of Arg-6 (positively charged) for Glu-6 (negatively charged) in pPLA2 reduced the activity of the enzyme on diC4PC by a factor of four (de Haas et al., 1987); Glu-71 may have a similar effect in bPLA2. The affinity of PLA2 for the lipid-water interface can be increased by the addition of negatively charged surfactants to the micelle or vesicle (Volwerk et al., 1986). This also seems to be the case for N71E, as in the absence of detergent the activity of the mutant was 3-4 fold lower than that of bPLA2 on micelles of diC4PC, but with mixed micelles (using deoxycholate) the enzyme activities were similar (Fig. 6.20). This suggests that deoxycholate could enhance the binding of N71E to the interface (by electrostatic interactions) probably by shifting the $E \leftrightarrow E^*$ equilibrium to favour $E^*$, thus overcoming the effect of the N71E substitution. This was a surprising observation, as the simple complementary charge model of enzyme-aggregated substrate binding would have predicted that the presence of the negative charged Glu-71 at the IRS would have prevented N71E binding to negatively charged micelles due to the unfavourable charge-charge interactions at the lipid-water interface.

The most unexpected observations were the catalytic rates measured for N71E with negatively charged substrates. The pancreatic PLA2s have a very high affinity for anionic compared to zwitterionic interfaces ($K_d \approx 0.1$ pM for diMPM compared to 1 mM for diMPC vesicles [Jain and Berg, 1989]). The results which are shown in Fig. 6.19 show that the mutant displays a $\approx 60\%$ (diMPG) and $\approx 50\%$ (diMPM) increase in activity compared to native bovine PLA2. The reason for this enhanced activity with negatively charged phospholipids may be due to a shift in the $E \leftrightarrow E^*$ equilibrium. With the addition of a negative charge to the lipid-water interface, a greater proportion of the enzyme is in the productive-binding $E^*$ form, which is reflected in the slight increase in activity. Again, this enhanced binding would not have been predicted by complementary charge model of binding. A reduction in activity may have been expected due to the decrease in affinity of PLA2 for the negatively charged interface caused by the unfavourable negative charge-charge interactions. Therefore the results clearly show that the mutant has a preference for anionic phospholipids and interfaces.
Chapter 6: Kinetic characterisation of bPLA2 mutants.

It seems that Asn-71 plays a key role in the formation of the IRS through its hydrogen bonding contributions to Ala-1, Tyr-73 and Asp-66. The loss of these interactions may prevent the formation of an ordered N-terminal α-helix and 58-71 surface loop, which is now unable to form the IRS and recognise organised lipid-water interfaces (as is seen for the zymogen). Alternatively, the reduction in binding to zwitterionic interfaces may be due to the introduction of a negative charge at the IRS by Glu-71 thus reducing electrostatic interactions with the micelle. The addition of a net negative charge (via an anionic detergent) to the micelle-interface enhances the enzyme's affinity for micelles, thus overcoming the effects of the mutation. In terms of the E↔E* equilibrium, the E form of the mutant enzyme predominates in the presence of zwitterionic micelles, thus only low turnover rates are observed. The activated form of the enzyme (E* which possesses a greater affinity for the aggregated substrate) only dominates when a negative charge is present at the lipid water interface and under these conditions the mutant enzyme displays high turnover rates.

6.11 V63FN71E bovine PLA2.

The final mutant made was the double mutant V63FN71E PLA2 resulting in changing the amino acid sequence of the 58-71 surface loop of bPLA2 to that found in pPLA2.

V63FN71E displayed some interesting kinetic and binding characteristics. The affinity for the enzyme for a variety of surfactants is summarised below.
Initial experiments showed that the double mutant had wild type binding characteristics at low pH with C16PN and similar activity to wild-type with the egg-yolk lecithin assay. But the effect of the mutations was more prominent at pH 8: with C16PN micelles there was a change for the affinity of the interface, with a 3 fold decrease in the value for $N-K_d$. The other pure micellar assays showed that, again like N71E, the double mutant had a lower affinity for the lipid water interfaces, but not to the same extent as N71E. Thus it seems that the deleterious effect of the N71E mutation can be slightly reversed by the presence of the V63F substitution. The low affinity for zwitterionic micelles (diC8PC) by the double mutant is similar to that of N71E. But again the enzymes binding properties can be enhanced by the presence of the negatively charged detergent deoxycholate (Fig. 6.23).
Chapter 6: Kinetic characterisation of bPLA₂ mutants.

Fig. 6.23: The turnover rates (s⁻¹) of authentic PLA₂s and N71E on a variety of phospholipids. The bars represent □ bPLA₂, □ pPLA₂ and V63FN71E.

The active site of the double mutant has also been affected, but not to the same extent as N71E, as the turnover rates with monomeric diC₆PC are higher (see Table 6.6). The specificity constant for diC₆PC micelles was 9 fold lower than native bPLA₂, reflecting the mutants poor affinity and turnover rate on micelles of diC₆PC (Fig. 6.14). The activity on mixed micelles was found to be intermediate to that of the single mutants V63F and N71E. There was also a significant increase in the catalytic rate with negatively charged substrates compared to the native enzyme, but again the values are between those rates measured for the single mutants.

The ability of the double mutant to counteract some of the effects of the N71E substitution may be due to the possible conformational change caused by the V63F mutation.
Chapter 6: Kinetic characterisation of bPLA₂ mutants.

The 58-71 surface loop region and the N-terminus are thought to be disordered when the enzyme is free in solution. It has already been discussed how the N71E mutation might prevent the stabilisation of the loop on micellar binding. Thus the slight increase in the ability of the double mutant to "recognise" organised lipid water interfaces in the absence of anionic detergents, may be explained by the effect on the loop stabilisation by the predicted conformational change caused by V63F. The V63F mutation in the double mutant may allow a more favoured packing arrangement of the side chains to a more "porcine like" conformation when the protein is at the interface. Also the effect of the N71E mutation, which may cause the loss of several important hydrogen bonds, may be compensated for by the enhanced hydrophobic interactions of the Leu-64 and Val-65 side chains, which are forced towards the IRS by the V63F mutation. Generally the double mutant seems to have intermediary characteristics of the single mutants for catalysis and micellar binding.

6.12 An engineered secondary calcium binding site?

Numerous experiments have shown that the porcine and the equine pancreatic enzymes possess a secondary low affinity Ca²⁺ binding site which is required for the enzyme to bind to neutral micellar interfaces at high pH (van Scharrenburg et al., 1984; Donné-Op den Kelder et al., 1984; van den Bergh et al., 1989). The amino acid residues which make the low affinity calcium binding site are the side chain carboxylate of Glu-71 and to a lesser extent Asp-66, as determined by biochemical/mutagenesis methods (Donné-Op den Kelder et al., 1983). Crystallographic data also suggests that the Glu-92 side chain carboxylate and Ser-72 backbone carbonyl may be involved (Dijkstra et al., 1983) (see Fig. 6.24).
Chapter 6: Kinetic characterisation of bPLA$_2$ mutants.

Fig. 6.24: The low affinity secondary Ca$^{2+}$ binding site. The bovine (magenta) and porcine (blue) structures have been superimposed. The CPK spheres represent the calcium atoms.

It seems conceivable that electrostatic forces (involving the side chains of Asp-66, Glu-71 and to a lesser extent Glu-92) are involved in determining the configuration of the 58-71 surface loop in pPLA$_2$. Glu-71 of pPLA$_2$ has an abnormally high pK$_a$ of 6.3 (van Scharrenburg et al., 1984; Donné-Op den Kelder et al., 1983) and thus will be protonated at pH of 5 (Fig. 6.25a). Therefore at pH 5, Glu-71 is able to stabilise the region around 58-71 by the charged hydrogen bond to Asp-66, which is assumed to have a lower, more normal pK$_a$. Thus even in the absence of calcium, pPLA$_2$ is able to bind to neutral zwitterionic micelles at low pH (van den Bergh et al., 1989). At pH 8 there will be a repulsive force between the negative charges (from Glu-71 and Asp-66) which prevents binding to neutral zwitterionic micelles in the absence of Ca$^{2+}$, perhaps by the localised distortion of the three dimensional structure (Fig. 6.25b). Binding of a second calcium ion and neutralisation of the negative
Chapter 6: Kinetic characterisation of hPLA₂ mutants.

Charges might, subsequently, induce an altered loop conformation that brings Asp-66 into the vicinity of Glu-71 (Fig. 6.25c). Thus the secondary calcium is essential to neutralise this charge and allow the enzyme to bind to micelles.

![Diagram of possible interactions of carboxylate groups at the second calcium binding site of pPLA₂ over a range of pHs and calcium concentrations.](image)

Fig. 6.25: The possible interactions of carboxylate groups at the second calcium binding site of pPLA₂ over a range of pHs and calcium concentrations.

Therefore, for the porcine enzyme, the electrostatic contribution of the weakly bound secondary calcium is essential for interfacial recognition (Scott et al., 1994). This secondary calcium site is absent from the bovine protein, as residue 71 is an Asn. Thus the bovine enzyme is able to bind to neutral zwitterionic micelles over a wide pH range. Therefore the question is, has the introduction of Glu-71 engineered a second calcium binding site into the enzyme? Generally, the affinity for zwitterionic lipid-water interfaces by PLA₂ can be improved by introducing a positive charge into the IRS of the protein, or a negative charge to the interface of the micelle (Scott et al., 1994; van Scharrenburg et al., 1981; van Scharrenburg et al., 1983; de Haas et al., 1987). Thus it may well be expected that the N71E mutant could cause the observed reduced affinity for zwitterionic micelles because of the presence of the negative charge, but this does not explain the reduced hydrolysis rates on monomeric substrates. V63FN71E or N71E probably do not bind a second calcium, since even in the presence of excess calcium (50 mM), high affinity binding to neutral micelles (C₁₅PN) is not observed at high pH values (Tables 6.2 and 6.3, Fig. 6.18). It seems that contributions from other residues or a subtle structural difference may be required to form a functional secondary calcium binding site in the single or double mutant. This is supported by the fact that the isoenzyme of pPLA₂ is also unable to bind a secondary calcium. The
isozyme only differs from the native pPLA$_2$ by four amino acids: Ala-12, His-17, Met-20 and Glu-71 in pPLA$_2$ and Thr-12, Asp-17, Leu-20 and Asn-71 in the isozyme. The inability of the isozyme to bind a secondary calcium was attributed to the lack of a carboxylate pK$_a$ of approximately 6.3 from Glu-71 (van den Bergh et al., 1989). The reason for the abnormally high pK$_a$ for Glu-71 was attributed to the local environment around the carboxylate, which was thought to be either strongly hydrophobic or negatively charged in nature (Donné-Op den Kelder et al., 1983).
CHAPTER 7

SUMMARY AND CONCLUSIONS

7.1 Maintaining solubility of recombinant proteins in E. coli.

Protein aggregation during overexpression in E. coli is common, since the environment of the prokaryotic cell cytoplasm does not provide the optimum conditions required for polypeptide folding. The key problem is that many overexpressed proteins are present at concentrations which are orders of magnitude greater than their normal expression levels. Thus folding intermediates will also be present at very high concentrations and so there will be a greater tendency for aggregation (Wall and Plückthun, 1995).

A number of new strategies have been developed to avoid inclusion body formation, thus producing soluble recombinant proteins with native-like biological activity directly from E. coli. These methods have advantages such as the reduction in time and costs of recombinant protein production. With soluble recombinant protein there is no need for in vitro refolding and the problems associated with down stream processing, such as purifying the protein with the native conformation. Current methods of producing soluble protein are summarised below.

7.1.1 Expression as a fusion protein.

This strategy involves fusing the gene of interest to a second "carrier" or "partner" gene to produce a fusion protein. Using this approach has other advantages such as the development of generic protein purification schemes (e.g. using a glutathione-S-transferase carrier protein and isolating the fusion protein with a glutathione affinity column), and can also eliminate the variability in expression yields (LaVallie and McCoy, 1995). The soluble recombinant protein of interest is released using a specific protease such as factor Xa. A wide range of fusion partners have been used, such as staphylococcal protein A to solubilise insulin-like growth factor I (IGF-1) (Samuelsson et al., 1994; 1991) and thioredoxin fused to a
variety of mammalian cytokines and growth factors (LaVallie et al., 1993). The reasons for their success in preventing inclusion body formation is still unclear, but it is thought that the high stability and solubility of the fusion partner may act as a covalently linked chaperone. Thus the carrier protein may allow the fused polypeptide chain to fold correctly and maintain its solubility.

7.1.2 Co-expression of "helper" proteins.

This method requires that modulators of protein folding are co-expressed at increased levels within the cell cytoplasm. The cellular levels of these "helper" proteins is usually low under normal physiological conditions, and so upon overexpression of the recombinant polypeptide the cellular systems usually become overwhelmed and aggregation of the folding intermediates occurs. With the increased levels of cellular helpers the recombinant polypeptide is maintained in a soluble conformation during folding and so preventing the formation of inclusion bodies. Examples of co-expression of modulators of folding are numerous, for example the most common proteins expressed are the molecular chaperones GroEL/ES and DnaK and DnaJ. The co-expression of GroEL/ES has been shown to increase the solubility of several recombinant tyrosine kinases (Amrein et al., 1995), and similar effects of GroEL/ES overproduction on solubility of the E. coli glutamate racemase protein (Ashiuchi et al., 1995) and S1 dihydrofolate reductase from Staphylococcus aureus (Dale et al., 1994) have also been noted. The co-expression of the Sec translocase proteins has also been shown to improve the secretion of recombinant polypeptides into the periplasmic space (Pérez-Pérez, et al., 1994). Localisation of recombinant proteins into the periplasm is useful as there are fewer proteases present therefore preventing protein degradation. Another advantage of targeting polypeptides to the periplasm is that disulphide bond formation in the recombinant protein can be favoured under the right conditions (see below). In order for the polypeptide to enter the secretory pathway it must possess the appropriate signal sequence and be maintained in soluble form, otherwise inclusion body formation within the cytoplasm will occur. For this reason overexpression of molecular chaperones such as DnaK and DnaJ, which have the ability to maintain the solubility of the polypeptide to be translocated, are known to
improve secretion (Pérez-Pérez et al., 1995). Alternatively increasing the levels of proteins directly involved in protein translocation (the membrane associated Sec translocase proteins), can significantly increase the processing efficiency as in the case of human interleukin-6 (Pérez-Pérez et al., 1994).

7.1.3 Bisulphide bond formation.

The need for disulphide bond formation in the target protein introduces a further complication and requires a combination of the above strategies to achieve the production of soluble folded polypeptide with the correct disulphide bridging pattern as found in the native protein. As the periplasmic space is the cellular location of disulphide bond formation within prokaryotes, the recombinant polypeptide is usually targeted here using the appropriate secretory signal. Disulphide bond formation can be favoured by the co-expression of the E. coli Dsb proteins and/or by changing the redox environment of the periplasm by adding oxidized and reduced glutathione to the culture medium. For example, the amount of correctly folded human IGF-1 isolated from the staphylococcal A fusion protein present in the periplasm was increased by the addition of reduced and oxidized glutathione to the growing culture (Samuelsson et al., 1996). The low molecular weight of the reduced and oxidized glutathione allowed them to diffuse into the periplasmic space. Thus once the fusion protein was present in the periplasm, the conditions allowed the formation and shuffling of the three disulphide bonds. α-Amylase/trypsin inhibitor from Eleusine coracana Gaertneri contains five intramolecular disulphide bridges. When overexpressed in the periplasm of E. coli the protein forms large amounts of misfolded inhibitor with incomplete or incorrect disulphides (Wunderlich and Glockshuber, 1993). The level of correctly folded protein increased 14-fold by co-expressing DsbA in conjunction with the addition of reduced glutathione to the growth medium (Wunderlich and Glockshuber, 1993). By using DsbA as a carrier protein for bovine enterokinase (which possess four intramolecular disulphide bonds), it was possible for active enzyme to be isolated from the periplasm of E. coli, once the partner enzyme had been enzymatically cleaved (Collins-Racie et al., 1995). In this case the DsbA functioned as a molecular chaperone to maintain the solubility of the recombinant polypeptide, but also
Chapter 7: Summary and conclusions.

Chapter 7: Summary and conclusions.

participated in disulphide bond formation and reshuffling. Disulphide bond formation has been shown to be possible within the cytoplasm of the bacterial cell, and was achieved by deleting the cellular gene encoding thioredoxin reductase ($\Delta trxB$) (Derman et al., 1993). The physiological role of thioredoxin reductase is to maintain thioredoxin in its reduced state, which in turn prevents the formation of disulphide bonds within the cell cytoplasm. It was shown that murine urokinase, which has six disulphide bonds, could now fold correctly with native like activity within the cytoplasm of the E. coli $\Delta trxB$ strain (Derman et al., 1993). A combination approach, using a DsbA carrier protein with pbPLA$_2$ overexpressed in the cytoplasm of a E. coli $\Delta trxB$ strain, allowed the formation of disulphide bonds within recombinant bPLA$_2$ thus giving native enzymatic activity (Mallinder et al., 1995 unpublished work). Therefore it is possible to fold proteins with multiple disulphide bonds within the cytoplasm of E. coli.

7.2 Overexpression of recombinant PLA$_2$.

The work presented in this thesis has shown that the problems associated with expression of ompT-pbPLA$_2$ in E. coli can be overcome. Only by inducing protein expression once the bacterial culture had reached the stationary growth phase at low temperatures and in minimal media was the majority of the recombinant polypeptide correctly processed to form inclusion bodies of pbPLA$_2$ within the periplasm. It was speculated that a heat shock response may be able to maintain the solubility of the partially folded recombinant polypeptide allowing it to enter the secretory pathway.

Thus it appears that protein secretion can be increased from E. coli without any further genetic manipulation. There is no need for the co-expression of the chaperone proteins to maintain the solubility of the recombinant polypeptide prior to secretion, or the overexpression of the Sec-machinery to improve translocation. The bacteria's own stress response is able to prevent premature aggregation of the recombinant polypeptide within the cytoplasm. This method might be generally applicable to increase the translocation efficiency of other recombinant proteins, but may also be dependent on the characteristics of the target polypeptide. For example, this methodology allowed bPLA$_2$ to be processed correctly but
Chapter 7: Summary and conclusions.

ompT-sPLA₂ still aggregated within the cytoplasm of the E. coli cell. This may have been due to the highly cationic nature of the protein (pI=10.5), which prevented its translocation against the proton-motive force, or the polypeptide chain may preferentially form insoluble aggregates before the heat shock proteins are able to intervene. Therefore highly insoluble proteins such as ompT-sPLA₂ will still form cellular inclusion bodies and so fail to enter the secretory pathway.

Secretion of sPLA₂ from MEL cells was found to be very low (0.5 mg/L culture), and this made it very difficult to isolate the enzyme in any appreciable amounts. The intrinsic insolubility, cationic charge and folding difficulties may have been responsible for the poor levels of enzyme secretion observed from MEL cells and yeast. The yeast based expression system which was used to express psPLA₂, showed that psPLA₂ did not fold within the cell, but instead formed an insoluble precipitate at or within the ER. This may be due to the N-terminal factor X₄ pro-sequence interfering with the folding of the polypeptide chain. The importance of a free N-terminus for folding has also been observed for enterokinase, where the first amino acid forms a salt bridge with the interior of the enzyme (Collins-Racie et al., 1995). The presence of any amino acid sequence which prevents this interaction (with the exception of DsbA) was found to prevent folding (Collins-Racie et al., 1995). Alternatively, it is possible that the secretory pathway had become saturated by the high level of psPLA₂ expressed. Thus with no increase in the enzymes responsible for disulphide bridge formation/folding within the ER, the majority of the protein within the ER fails to fold and forms the observed insoluble precipitate.

7.3 In vitro refolding.

The production of a specific protein in high yields is not just of interest in academic research, where the goals are to understand the proteins structure, function and possible involvement in the onset and progression of a specific disease. With advancements in molecular biology and biotechnology any protein can be overexpressed in a variety of cellular expression systems, as long as the gene sequence is available. Thus the large scale production of specific proteins of therapeutic use have become of vital importance to the biotechnology
industry, for example the market for cytokine products which involve an in vitro protein refolding stage is greater than £500 million (Thatcher and Hitchcock, 1994). The organism of choice is still bacteria, as they are easy to culture and their genetics are well characterised.

In vitro refolding is a crucial process in the production of a large number of foreign proteins which when overexpressed in *E. coli* initially form insoluble inclusion bodies. The removal of molecular oxygen from the refolding buffer was shown here to improve the folding efficiency of pro-bPLA$_2$ by approximately 83%. It was presumed that the molecular oxygen caused a greater proportion of the disulphide bonds formed within the protein to be incorrect. The presence of molecular oxygen may favour the formation of disulphide bonds that trap the protein into a conformation where the molecule is more susceptible to aggregation, or the conformation of the polypeptide chain may prevent the rearrangement of the disulphide bonds as they have become inaccessible to reduced glutathione. Thus the removal of oxygen may allow the cysteine residues to become oxidised in a more controlled manner with reduced and oxidised glutathione. This unique approach improved the amount of bPLA$_2$ with the native-like disulphide bridging pattern, but it may be that this methodology is specific to PLA$_2$ and may not be applicable to other proteins containing disulphide bridges. The in vitro refolding of any significant amounts of psPLA$_2$ overexpressed in *E. coli* was impossible using the strategies described in Chapter 3. The protein was expressed to a high level within the cell cytoplasm, but it appeared that refolding sPLA$_2$ with an N-terminal extension prevented the protein achieving its native structure.

After in vitro refolding a purification procedure must be devised that can remove incorrectly folded species, and a method must be available to determine when a homogeneous sample has been achieved. The use of several columns steps to purify recombinant bPLA$_2$ was essential to produce a homogenous protein sample for structural and kinetic studies. The consequences of using an inadequate protocol to remove incorrectly folded protein conformers can be seen from the work carried out by Liu *et al.*, (1995) with recombinant mutants of bPLA$_2$. The nmr spectra of the mutant enzymes which had been overexpressed in *E. coli* and refolded in vitro, clearly showed that other protein conformers were present in the sample. This suggested that the two column purification step used (Fast Flow Q-Sepharose
Chapter 7: Summary and conclusions.

followed by Fast Flow S-Sepharose) was insufficient to purify the enzyme to homogeneity (Noel et al., 1991). The quality of the nmr spectra produced after the purification method outlined in Chapter 2 (Sections 2.5.2 to 2.5.5) was superior to those previously published (Liu et al., 1995).

The combination of improved translocation efficiency by the late induction of protein expression and the oxygen-free refolding protocol, significantly improved the final amount of recombinant isolated. A 70% increase in recombinant protein production was achieved by the methods outlined in this thesis, i.e. 22.1 mg/L of bacterial culture for N71E compared to the previously highest reported amount of 12.8 mg/L of culture for porcine PLA₂ (van den Berg et al., 1995).

7.4 The 58-71 surface loop of bPLA₂.

Fig. 7.1 shows a comparison of the results from all the binding and kinetic assays of the authentic and recombinant bPLA₂. From these results, the following general conclusions can be drawn. The 58-71 surface loop is crucial to both interfacial recognition and catalysis as it contributes several residues to the IRS and the vital hydrogen bond network involving the N-terminus and the active site. The results presented are consistent with the fact that both electrostatic and hydrophobic interactions play a key role in interfacial recognition and that no one residue is responsible for the observed differences in binding and catalysis between bovine and porcine PLA₂. Thus even though there was a significant increase in affinity for neutral zwitterionic lipid-water interfaces with the introduction of a single point mutation in V63F, the increased binding was not caused by Phe-63 interacting directly with the micelle interface, but rather by the proposed conformational change in the 58-71 surface loop. The mechanism by which this occurred may be through a structural change in the 58-71 surface loop, brought about by Phe-63. This structural change may force hydrophobic residues (such as Leu-64 and Val-65) towards the IRS, thus improving interactions with the surface of the micelle. In the presence of a lipid-water interface the E⁺ form of the enzyme is favoured in the E ←→ E⁺ equilibrium. Therefore the surface loop may have a conformation more similar to that of pPLA₂ than bPLA₂, thus explaining the significant change in the binding
Chapter 7: Summary and conclusions.

characteristics of the V63F mutant, but the V63F mutant is still catalytically more similar to bPLA2. It appears that Phe-63 is indirectly responsible for the observed difference in affinity for the lipid-water interface between bovine and porcine PLA2, as this residue controls the structural conformation of the 58-71 surface loop. Therefore, the results of the V63F bPLA2 mutation differ from the conclusions for the porcine mutant F63V (Thunnissen et al., 1993). It was found that the F63V mutation altered the conformation of the 58-71 surface loop so that it was similar to the structure found in bPLA2, but the mutant was still kinetically more similar to pPLA2 than bPLA2. Thus it was concluded that Phe-63 was not solely responsible for the binding differences between the bovine and porcine enzymes. The effects of F63V in pPLA2 may be difficult to assess as the remaining 18 amino acid residues which differ between the two proteins, may also contribute to interfacial binding. By carrying out the reverse experiment in bPLA2 (i.e. V63F) the contribution of the residue at position 63 has been clarified.

The Asn-71 to glutamate substitution introduces an extra negative charge at the IRS. With zwitterionic micelles of C16PN (pH 8) or diC8PC, N71E and V63FN71E have a very low affinity for the lipid water interface. Therefore Glu-71 may prevent binding to zwitterionic interfaces purely due to the presence of the negative charge at the interface, thus reducing the electrostatic contribution of the 58-71 loop to interfacial binding (i.e. the E form of the enzyme is favoured in the $E = \rightleftharpoons E^*$ equilibrium). Alternatively, it may be that the substituted Glu-71 destabilises the 58-71 as the longer side chain and negative charge is difficult to accommodate within the molecule and causes the loss of the hydrogen bond interactions with Ala-1, Try-73 and Asp-66 (Fig. 6.21). Therefore this mutation would affect both the 58-71 surface loop and also the N-terminal helix, both regions which require to be ordered to form the IRS. The inability to form ordered structures when at the lipid-water interface would prevent binding to zwitterionic interfaces (i.e. the E form of the enzyme would predominate), thus reducing the observed $k_{cat}$. Catalysis on monomeric substrates has been reduced by 50%, but this may not be significant.

The Phe mutation in V63FN71E appeared to be able to compensate for some of the reduction in binding to the micellar interface observed with the single N71E mutant (at pH 5 with C16PN). This is probably because Glu-71 will be protonated and because of the increased
hydrophobic interactions from Leu-64 and Val-65 due to the conformational change of the 58-71 surface loop. Therefore the binding characteristics of V63FN71E will be similar to the wild type enzyme. Under assay conditions at which the pH is 8.0, the double mutant behaves very similarly to the N71E mutant. This may be as a consequence of the Glu-71 now being deprotonated and so as discussed above (also Section 6.10), this may cause the destabilisation of the N-terminus and the 58-71 surface loop, preventing the formation of the IRS. The presence of Phe-63 reduces the effects of Glu-71 on binding and catalysis only marginally. Unexpectedly, the addition of a negative charge to the micellar interface (using a negatively charged phospholipid or detergent), seemed to enhance the binding of N71E and V63FN71E to the interface. Considering that Glu-71 would be deprotonated under the assay conditions, this should reduce the binding to negatively charged interfaces due to charge-charge repulsion. However from the diMPM, diMPG and diCgPC assays done in the presence or absence of 15 mM deoxycholate, it was shown that, for both N71E and V63FN71E, the E* form of the enzyme were favoured when the surfactant was present at the micelle interface. Therefore the simple complementary charge model of interfacial binding is insufficient to explain the above results, as it would have been predicted that N71E and V63FN71E would have a low binding affinity for negatively charged phospholipid aggregates due to the unfavourable electrostatic interactions.

The mutants containing N71E, unlike V63F, did not impart any obvious characteristics of the porcine enzyme to the bovine mutants. The results did suggest that Asn-71 is important both to interfacial recognition and indirectly to catalysis, possibly through the hydrogen bonds which involve this residue and the stabilisation of regions of secondary structure such as the N-terminus, the primary calcium binding loop and the 58-71 surface loop, which form the IRS. This supports the recent nmr work carried out by van den Berg et al., (1995), who observed that the structure of pPLA2 had regions of disorder (e.g. the N-terminal helix), and so low enzymatic activity. Upon binding to a micellar interface, these regions become ordered so that the residues involved in the IRS and active site are optimally arranged for catalysis, and so an increase in activity is observed (van den Berg et al., 1995a: 1995b). The kinetic assays on N71E and V63FN71E with zwitterionic micelles, showed that there was a shift in the $E \rightleftharpoons E^*$ equilibrium, so that the E form of the enzyme predominates. Structural
information either from nmr or X-ray crystallography is essential to confirm the proposed mechanisms by which bPLA$_2$ has been affected structurally by the Val-63 and Asn-71 substitutions. This may involve using nmr to determine the structure of both the enzyme free in solution and in the ternary complex (the protein bound to a non-hydrolysable micelle with the active site occupied by an inhibitor), as previously done for pPLA$_2$ by van den Berg et al., (1995a; 1995b) with pPLA$_2$. If determined by X-ray crystallography, the structure of the mutant enzymes would be similar to that of the protein at the lipid water interface.
Chapter 7: Summary and conclusions.

Fig. 7.1: Summary of the binding and kinetic data of authentic procine, bovine, V63F, N71E and V63FN71E PLA$_2$.
APPENDIX A

GENERAL METHODS

A1 MICROBIOLOGY CULTURE METHODS.

A1.1 Media.

All solutions used for bacterial work were either autoclaved (120°C for 20 minutes), filter sterilised using sterile filter units (50-250 mL) or filtered using 0.2 μm Acrodiscs filters.

A1.2 Agar plates.

100 mL of 3% agar (w/w) and 100 mL of 2 x A salts (see A1.3 for composition) were autoclaved separately in 200 mL Duran bottles and left to cool. When required, the agar was heated until molten and left to cool but not set. To the 100 mL of 2 x A salts the following components were added:

- 80 μL 500 μg/mL biotin (filter sterilised)
- 0.4 mL 1 M MgSO₄ (autoclaved or filter sterilised)
- 20 μL 1 M CaCl₂ (autoclaved or filter sterilised)
- 10 mL 20% glucose (autoclaved)
- 200 μL 100 mg/mL ampicillin in 50% ethanol (filter sterilised)
- 100 μL 100 mg/mL chloramphenicol in 100% ethanol (filter sterilised)

When the agar had cooled to below 45°C, the 2 x A salts mixture was added and mixed. The A salts agar was then poured into sterile Petri dishes and left to set. Once set the plates were dried at 50°C for 5 minutes. M9 agar plates were prepared as above using 2 x M9 media (see A1.3 for composition). Luria Bertini (LB) and 2 x YT agar plates were made by adding agar to 1.5% (w/w) to a given volume of media before autoclaving.
A1.3 Culture Media.

A salts minimal media were used for the expression of bPLA$_2$ and its mutants, as well as for isotopic labelling with $^{15}$N. 10.5 g $K_2$HPO$_4$, 4.5 g $KH_2$PO$_4$, 1.0 g $NH_4$Cl and 0.25 g tri-sodium citrate was added to 1 L of distilled water, divided into 250 mL per 2 L baffled flask and autoclaved. Prior to inoculation with cells, the following components were added to each flask:

- 100 µL 500 µg/mL biotin (filter sterilised)
- 0.5 mL 1 M MgSO$_4$ (autoclaved or filter sterilised)
- 25 µL 1 M CaCl$_2$ (autoclaved or filter sterilised)
- 12.5 mL 20% glucose (autoclaved)
- 250 µL 100 mg/mL ampicillin in 50% ethanol (filter sterilised)
- 125 µL 100 mg/mL chloramphenicol in 100% ethanol (filter sterilised)

M9 minimal media were prepared using 6.0 g $Na_2$HPO$_4$, 3.0 g $KH_2$PO$_4$, 0.5 g sodium chloride and 1.0 g $NH_4$Cl per litre of distilled water. Following autoclaving, the components listed below were added per 250 mL flask:

- 1 mL 1 M MgSO$_4$ (autoclaved or filter sterilised)
- 50 µL 1 M CaCl$_2$ (autoclaved or filter sterilised)
- 5 mL 20% glucose (autoclaved)
- 250 µL 100 mg/mL ampicillin in 50% ethanol (filter sterilised)
- 125 µL 100 mg/mL chloramphenicol in 100% ethanol (filter sterilised)

The components of LB and 2 x YT are given below and were made up to 1 L using distilled water.

<table>
<thead>
<tr>
<th>2 x YT/per L</th>
<th>LB/per L</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 g tryptone</td>
<td>10 g tryptone</td>
</tr>
<tr>
<td>10 g yeast extract</td>
<td>5 g yeast extract</td>
</tr>
<tr>
<td>5 g sodium chloride</td>
<td>10 g sodium chloride</td>
</tr>
</tbody>
</table>
Appendix A

A2 DNA MODIFICATION REACTIONS.

A2.1 PCR conditions.

The reactions were carried out in small Eppendorf tubes (500 μL) using a Perkin-Elmer Thermocycler and contained the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration/Unit</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Deep Vent/Taq</td>
<td></td>
<td>10 μL</td>
</tr>
<tr>
<td>reaction buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dNTPs</td>
<td>200 μM each of ATP, GTP, CTP and TTP</td>
<td></td>
</tr>
<tr>
<td>(final concentrations)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Template DNA</td>
<td>25-100 ng</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.2-0.4 μM</td>
<td></td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.2-0.4 μM</td>
<td></td>
</tr>
<tr>
<td>Deep vent/Taq</td>
<td>1-2.5 units</td>
<td></td>
</tr>
<tr>
<td>polymerase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterile water</td>
<td>volume to 100 μL</td>
<td></td>
</tr>
</tbody>
</table>

The reaction mixture was layered with 50 μL of mineral oil.

A2.2 Digestion of DNA with restriction enzymes.

2-6 μg of DNA, 3 μL 10x restriction buffer and water (to give a final reaction volume of 30 μL) were transferred to a microcentrifuge tube. To ensure that the cleavage conditions are optimised for the restriction enzyme (i.e. 100% enzymatic activity), a specific 10x reaction buffer is provide by the manufacturer. 5 units of restriction enzyme (10 units/μL) were added, the solution mixed gently and then incubated for 3 hours at 37°C.

Double digests (the addition of two restriction enzymes together) were carried out using 3 μL of 10x universal restriction buffer, 5 units of each enzyme and the final volume of the reaction mixture was kept to 30 μL using distilled water.

Sequential digests were carried out when no common restriction buffer was available, or a particular enzyme could not cut close to the end of linear DNA (New England Biolabs catalogue). The DNA was cut first with the restriction enzyme which had the greatest activity
(as described above), purified using the agarose gel electroelution protocol (Appendix A3.4), and then digested with the second restriction enzyme.

When the digestion reaction was complete, the products of the digest were analysed by gel electrophoresis using the appropriate percentage agarose (see Table A.1).

A2.3 Alkaline phosphatase treatment of DNA.

Calf intestinal alkaline phosphatase (CIAP) was used to remove the 5' phosphate group from linearised vector DNA and therefore prevent recircularisation and religation of linearised vector DNA. Up to 10 pmole of 5' ends was added to 10 μL of 10x CIAP buffer, 5 μL of 0.1 units/μL CIAP and distilled water added to a final volume of 100 μL. The solution was incubated at 37°C for 60 minutes after which the reaction was stopped by adding 2 μL of 0.5 M EDTA. The solution was extracted with phenol/chloroform followed by precipitation with isopropanol.

A2.4 5' phosphorylation of DNA using T4 polynucleotide kinase.

T4 polynucleotide kinase (T4 PNK) was used to add a 5' phosphate group to DNA. This allowed a phosphodiester bond to be formed between the 3' OH and 5' phosphate by T4 DNA ligase. To 30 pmole of DNA, 10 units of T4 PNK, 2 μL of 10x T4 PNK buffer, ATP to a final concentration of 1 mM were added and the volume adjusted to 20 μL using distilled water. The mixture was incubated at 37°C for 30 minutes after which time the sample was phenol/chloroform treated and the DNA isopropanol precipitated.

A2.5 DNA ligations using T4 DNA ligase.

T4 DNA ligases was used to join DNA fragments having a 5' phosphate and 3' OH. The vector to insert ratio used in the ligation was 1:3, using up to 100 ng of total DNA (3-30 fmole of vector ends and 9-90 fmole of insert ends). The final reaction volume was adjusted to 20 μL using distilled water. Digested vector DNA and 4 μL of 5x DNA ligase buffer were
Appendix A

added to the water. The sample was heated to 65°C for 1 minute and then cooled on ice. Insert DNA and 100 units of DNA ligase were added and the reaction was allowed to proceed at 16°C overnight. The mixture was diluted five fold and 40 μL was used to transform competent E. coli DH5α cells.

A3 MOLECULAR BIOLOGY TECHNIQUES.

A3.1 DNA gel buffers.

TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA) buffers were routinely used for agarose gel electrophoresis and DNA sequencing. Stock solutions prepared were 10 x TBE and 50 x TAE, which were then diluted to give the desired concentrations. The components of the buffers are given below:

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x TBE</td>
<td>0.89 M Tris-HCl pH 8.0</td>
<td>2 M Tris base</td>
</tr>
<tr>
<td></td>
<td>0.89 M Boric acid</td>
<td>50 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>20 mM EDTA</td>
<td>1 M Acetic acid</td>
</tr>
<tr>
<td></td>
<td>Final pH=8.3</td>
<td>Final pH=8.3</td>
</tr>
</tbody>
</table>

A3.2 Agarose gel electrophoresis

DNA was routinely run on agarose gels to identify possible positive clones from restriction digests and to isolate DNA fragments. The percentage agarose used was dependent on the size of the DNA fragment to be visualised/isolated (Table. A.1)

For a 0.7% (w/w) gel, 0.7 g of ultra-pure agarose was mixed with 100 mL of 0.5 x TBE buffer in a conical flask and heated until the powder had dissolved. The solution was cooled under running water before 0.5 μL of ethidium bromide (10 mg/mL) was added. A gel casting bed was used to form and run the gel. The two open ends of the casting bed were sealed with masking tape, thus forming an enclosed container. The agarose solution was then poured into the gel casting bed and a comb (10 or 20 toothed) inserted and the agarose left to set. Once set (15-30 minutes depending on the % agarose), the masking tape and comb were
removed and the casting bed and gel lowered into the gel tank containing 0.5 x TBE buffer and 10 μL ethidium bromide (10 mg/mL). DNA samples were prepared by mixing 2 μL of DNA solution with 1 μL 10x loading dye and 7 μL of distilled water. As well as running the samples, molecular weight markers were also used at concentrations recommended by the manufacturer (lambda phage DNA cut with HinD III (Table A.2), or PCR DNA ladder). The gel was run at 150 V until the loading dye had migrated 3/4 the length of the agarose gel. The DNA was visualised using ultraviolet light and DNA concentrations and sizes estimated using the molecular weight markers. 5 μL of PCR ladder produces the following bands 1000, 700, 500, 400, 300, 200, 100 and 50 bp, each band containing 50 ng of DNA.

<table>
<thead>
<tr>
<th>Agarose gel (%)</th>
<th>Optimum resolution for linear DNA (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>30 to 1.0</td>
</tr>
<tr>
<td>0.7</td>
<td>12 to 0.8</td>
</tr>
<tr>
<td>1.0</td>
<td>10 to 0.5</td>
</tr>
<tr>
<td>1.2</td>
<td>7 to 0.4</td>
</tr>
<tr>
<td>1.5</td>
<td>3 to 0.2</td>
</tr>
</tbody>
</table>

Table A.1: The percentage agarose required to resolve various DNA fragments.

**A3.3 Lambda phage DNA cut with HinD III.**

<table>
<thead>
<tr>
<th>Fragment size (Kb)</th>
<th>Amount of DNA (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.13</td>
<td>477</td>
</tr>
<tr>
<td>9.42</td>
<td>194</td>
</tr>
<tr>
<td>6.56</td>
<td>135</td>
</tr>
<tr>
<td>4.36</td>
<td>90</td>
</tr>
<tr>
<td>2.32</td>
<td>48</td>
</tr>
<tr>
<td>2.03</td>
<td>42</td>
</tr>
<tr>
<td>0.56</td>
<td>12</td>
</tr>
<tr>
<td>0.125</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Table A.2: Restriction digest of lambda phage DNA with HinD III showing the resultant fragment sizes and amounts from 1 μg of DNA.
A3.4 Isolation of DNA from agarose gels.

DNA obtained from restriction digests and from PCR was isolated using the following protocol. The DNA samples were prepared and run on an agarose gel as described in Section A3.2. The gel was examined under ultraviolet light illumination and the portion of the gel containing the band which had migrated the correct distance (as judged against known molecular weight markers) was excised using a scalpel. The isolated gel fragment was placed in dialysis tubing (8 kDa molecular weight cut-off) and 500 μL of 0.5 x TBE buffer was added. DNA was eluted by electroelution at 150 V for 15 minutes after which the current was reversed for 10 seconds. The agarose fragment was then observed under UV illumination to check that the DNA had been eluted from the gel. DNA was isolated from the 0.5 x TBE buffer by precipitation with isopropanol and the pellet dried under vacuum for 1 minute to remove any residual ethanol.

A3.5 TE-saturated phenol/chloroform.

Equal volumes of phenol and TE (Tris-EDTA) buffer (1 mM EDTA and 10 mM Tris-HCl, pH 8.0) were mixed and the phases allowed to separate. The phenol lower phase was then removed and mixed with an equal volume of chloroform:isoamyl alcohol (24:1 v/v).

A3.6 TE-phenol/chloroform extraction of DNA.

To the DNA solution an equal volume of TE-saturated phenol/chloroform solution was added and vortexed for 1 minute. The solution was then centrifuged at 13,000 rpm for 2 minutes, after which the upper aqueous phase was mixed with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and centrifuged at 13,000 rpm for 2 minutes. Again the upper aqueous phase was removed and placed in a fresh tube and the DNA precipitated with isopropanol.
A3.7 Isopropanol precipitation of DNA.

The DNA was precipitated from solution by adding 1/5 of the volume of 5 M potassium acetate pH 4.5 followed by 2 volumes of ice-cold isopropanol. DNA was pelleted by centrifugation at 13,000 rpm for 30 minutes. The supernatant was removed by aspiration and the pellet washed with 70% ice-cold ethanol followed by centrifugation at 13,000 rpm for 5 minutes. The ethanol was then removed, the pellet dried under vacuum for 1 minute and then redissolved in 10-20 μL of sterile Super Q water.

A3.8 Plasmid mini preps.

Plasmid DNA was isolated from E. coli cells (DH5α) by several methods depending on the quantity and purity of the DNA required. Plasmid mini preps were used for the detection of positive clones by restriction enzyme digests and single base sequencing. A 5 mL culture (2 x YT) of cells containing the appropriate antibiotic was grown overnight at 37°C. 1.5 mL of culture was pelleted by centrifugation (13,000 rpm for 2 minutes) and resuspended in 100 μL of ice-cold GTE (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0) buffer. After 5 minutes at room temperature, 200 μL of 0.2 M NaOH, 1% SDS was added and the solution incubated on ice for 5 minutes. 150 μL of ice-cold potassium acetate pH 4.8 was added and the mixture vortexed. The mixture was centrifuged for 5 minutes at 13,000 rpm. The supernatant was added to a clean micro-centrifuge tube containing 1 μL (10 mg/mL) of RNase A and incubated at 37°C for 30 minutes. The plasmid DNA was then precipitated with isopropanol (Section A3.7).

DNA for sequencing was purified using QIAGEN-tip-100 columns (for up to 100 μg of plasmid DNA). Cells were grown in 150 mL LB media containing 100 μg/mL ampicillin and were grown overnight at 37°C. The plasmid DNA was purified as recommended by the manufacturer.
Appendix A

A3.9 Determination of DNA concentration by absorbance spectroscopy.

The concentration of DNA was determined by measuring the absorbance at $\lambda = 260$ nm ($A_{260}$):

- Concentration of double stranded DNA ($\mu$g/mL) = $A_{260} \times 50$
- Concentration of single stranded DNA ($\mu$g/mL) = $A_{260} \times 33$

The following values were used to calculate concentrations and amounts of DNA.

- The average molecular weight of a deoxynucleotide base = 324.5 Daltons
- 1 $\mu$g of 1,000 bp DNA = 1.52 pmoles (3.03 pmoles of ends)
- 1 pmole of 1,000 bp DNA = 0.65 $\mu$g

A3.10 Preparation of competent *E. coli* cells.

*E. coli* cells (from Novagen or Life Technologies Inc.) were streaked out onto a LB plate with the appropriate antibiotic (DH5α with no selection, BL21 (DE3) pLysS with 50 $\mu$g/mL chloramphenicol) and incubated overnight at 37°C. 250 mL of LB broth containing 20 mM MgSO$_4$ was inoculated with several colonies from the agar plate. The culture was grown at 37°C until the O.D$_{600}$ reached 0.48-0.50, after which the flask was chilled on ice for 5 minutes. All solutions and centrifuge tubes were kept on ice, as higher temperatures result in a reduction of cell competence. The cells were pelleted by centrifugation (5,000 rpm for 10 minutes at 4°C) gently resuspended in 100 mL Tfb I (see below) and then incubated on ice for 5 minutes. After incubation the cells were again pelleted by centrifugation (5,000 rpm for 10 minute at 4°C), gently resuspended in 10 mL of Tfb II (see below) and left on ice for 15 minutes. The cells were then aliquoted (100 $\mu$L) into precooled Eppendorf tubes (on dry ice) and stored at -70°C.

<table>
<thead>
<tr>
<th>Tfb I</th>
<th>Tfb II</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mM potassium acetate</td>
<td>10 mM MOPS (or PIPES)</td>
</tr>
<tr>
<td>100 mM RbCl$_2$</td>
<td>75 mM CaCl$_2$</td>
</tr>
<tr>
<td>10 mM CaCl$_2$</td>
<td>10 mM RbCl$_2$</td>
</tr>
<tr>
<td>50 mM MnCl$_2$</td>
<td>15% glycerol (v/v)</td>
</tr>
<tr>
<td>15% glycerol (v/v)</td>
<td>pH 6.5 with 1 M NaOH</td>
</tr>
<tr>
<td>pH 5.8 with dilute acetic acid</td>
<td></td>
</tr>
</tbody>
</table>
Both solutions were filter sterilised.

A3.11 Transformation of competent cells.

Competent cells were thawed on ice for 20 minutes. 40 μL of plasmid DNA (2.5 μg/mL) was added to the Eppendorf tube and the mixture incubated on ice for 30 minutes. The cells were then heat-shocked for exactly 90 seconds at 42°C and placed on ice for 2 minutes. 600 μL of LB media was added to the Eppendorf tube and the cells allowed to recover by incubation at 37°C for 1 hour with gentle shaking. Transformed cells were selected by plating out 100 μL of the culture on LB agar plates with the appropriate antibiotics.

A3.12 Preparation of glycerol stocks.

A single colony was picked from the agar plate and added to 5 mL of 2 x YT media containing the appropriate antibiotics. The culture was grown at 37°C in a shaking incubator (200 rpm) until the O.D._600 reached 0.5-0.8. To the culture, 50% sterile glycerol (autoclaved) was added until the final glycerol concentration was between 10-15%. The cells were then aliquoted into Eppendorf tubes which had previously been chilled on dry-ice and stored at -70°C.

A4 DNA SEQUENCING.

A4.1 Denaturing double-stranded plasmid DNA and primer annealing.

2 μL of 2 M NaOH was added to 8 μL of the plasmid DNA solution (2-3 μg of plasmid DNA) and incubated at 37°C for 10 minutes. All solutions were chilled on ice before use. To the tube 3 μL of 3 M sodium acetate pH 4.5, 7 μL of distilled water and 60 μL of isopropanol were added and incubated on dry-ice for 15 minutes. The sample was thawed out and centrifuged at 13,000 rpm for 10 minutes to pellet the DNA. The supernatant was removed and the pellet washed with 50 μL of 70% ethanol and recentrifuged at 13,000 rpm for 5 minutes. The ethanol was removed and the pellet dried under vacuum for 1 minute.

227
of distilled water, 2 μL of annealing buffer (280 mM Tris-HCl pH 7.5, 100 mM MgCl₂ and 350 mM sodium chloride) and 2 μL of sequencing primer (5 ng/μL) was added to the pellet. The Eppendorf tube was heated to 70°C and allowed to cool to room temperature.

A4.2 Dideoxynucleotide sequencing.

Plasmid DNA was made single stranded (using alkaline denaturation) and annealed with the appropriate primer for sequencing. ³⁵S-labelling and short termination reactions were carried out in a microtitre plate using the T7Sequencing kit from Pharmacia as recommended by the manufacturer.

Gel plates used for DNA sequencing were thoroughly cleaned with detergent and water; dried and cleaned with industrial methylated spirits (IMS); and finally cleaned with 100% acetone. The plates were separated with 0.4 mm spacers and then sealed along the sides and base with tape. One of the plates had previously been treated with repel-silane to prevent adhesion of the gel to the glass upon separation of the plates. The sides of the plates were clamped using large bull-dog clips. The polyacrylamide gel consisted of 9.5 mL of 40% acrylamide solution, 31.5 g of urea, 3.75 mL of 10 x TBE buffer made up to 75 mL using distilled water. Once the urea had dissolved the solution was degassed under vacuum using a 200 mL Buchner flask with rapid stirring for 5 minutes. 50 μL of 25% ammonium persulphate and 50 μL TEMED were added to the acrylamide mixture to initiate polymerisation. Using a 50 mL syringe the gel solution was introduced between the gel plates, taking care to avoid the introduction of air bubbles. After pouring the gel a comb was inserted 3 mm into the top of the plates, after which they were left in a horizontal position for the gel to set (usually 2-3 hours). The tape was removed from the base of the plates and the gel was then clamped to the gel stand. A salt gradient was set up using 1 M sodium acetate, 0.5 x TBE buffer as the lower buffer and 0.5 x TBE buffer as the top buffer. Prior to loading the samples the gel was prewarmed by running at 70 W, 2300 V for 1 hour. The termination reactions were heated to 80°C for 2 minutes and 2 μL of each mix loaded onto the in the order G, A, T, C. The gel was run at 70 W, 2300 V until the second dye front (cynanol green) had reached ¾ of the length of the gel. After running the gel, the plates were separated, the gel transferred to a sheet of
Appendix A

Whatman 3MM paper, covered with Saranwrap and dried down at 80°C for 1 hour. A sheet of autorad film was then exposed to the dried gel overnight (16-20 hours), after which the DNA sequence was read directly from the developed film.

A5 MISCELLANEOUS.

A5.1 Synthesis of disodium 2-nitro-5-thiosulphobenzoate (NTSB).

The method for the synthesis of disodium 2-nitro-5-thiosulphobenzoate (NTSB) from 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was taken from Thannhauser et al., (1984). 0.99 g of DTNB (50 mM final concentration) was dissolved in 50 mL of 1 M Na$_2$S and the pH adjusted to 7.5. The solution was placed in a water bath heated to 38°C and vigorously stirred as oxygen gas was bubbled through it. The reaction was judged to have gone to completion once the solution had change colour from red/orange to a pale yellow (usually 24 hours). The solution was aliquoted and stored at -20°C.

A5.2 Oxidation of reduced glutathione.

The protocol for the oxidation of reduced glutathione to form oxidised glutathione was modified from Thannhauser et al., (1984). 200 mL of 100 mM reduced glutathione, 200 mM (NH$_4$)$_2$HPO$_4$ pH 8.0 (using NH$_4$OH) was placed into a 2 L baffled flask and molecular oxygen sparged through the solution for 5 minutes. The flask was sealed using Nescofilm and placed in a gyratory shaker at 300 rpm at 37°C until all the reduced glutathione had been oxidised (usually 12-24 hours). Oxidation was monitored by using the Ellman assay which gives a colour change in the presence of free thiols (Creighton, 1989). 50 μL of 10 mM DTNB (in 0.1 M sodium phosphate pH 7.3) was mixed with 1 mL of test solution and the absorbance measured at 412 nm. If the absorbance was greater than 1, the test solution was diluted using distilled water. Using the absorption coefficient ε = 14,150 M cm$^{-1}$ the concentration of free thiols was calculated, and hence the percentage oxidation. The final concentration of oxidised
Appendix A

glutathione was ~50 mM, as ~99.5% of the reduced glutathione was oxidised. The solution was stored at 4°C and used within 2 weeks.

A5.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was carried out using the Bio-Rad Mini-PROTEAN II gel electrophoresis kit and protocols. A quarter volume of Sample Buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.025% w/v bromophenol blue) was added to the protein sample, mixed and then heated at 100°C for 4 minutes. The protein samples were loaded onto a 15% SDS polyacrylamide gel (containing 0.1% SDS) alongside standard molecular weight markers. The gel was run in 25 mM Tris-HCl, 192 mM glycine buffer pH 8.3, (1% SDS) at 200 V until the dye front had reached the bottom of the gel (approximately 45 minutes). The gel was stained with Coomassie Blue for 30 minutes, destained and then examined.

A5.4 Tris-Tricine SDS PAGE.

Tris-tricine gels (Schagger and von Jagow, 1987) were used to resolve low molecular weight proteins. Gels were run using the Bio-Rad Mini-PROTEAN II gel electrophoresis kit. The methodology was as described for standard SDS-PAGE but with the following modifications. Sample buffer consisted of 4% SDS (w/v), 12% glycerol (v/v), 50 mM Tris-HCl pH 6.8, 2% β-mercaptoethanol (v/v) and 0.01% Coomassie Blue R250 (w/v). The separating gel (16.5% acrylamide) consisted of 5.5 mL of 30% acrylamide, 1.1 mL 100% glycerol and 3.47 mL of gel buffer (3 M Tris-HCl pH 8.45, 0.3% SDS). The stacking gel (3.9% acrylamide) consisted of 1.3 mL of 30% acrylamide, 2.5 mL of gel buffer and 6.2 mL of distilled water. The acrylamide was polymerised by the addition of 50 µL of 10% ammonium persulphate and 15 µL of TEMED.

The protein samples were separated using 0.2 M Tris-HCl pH 8.9 as the anode (outer) buffer and 0.1% SDS (w/v), 0.1 M Tris-HCl pH 8.45, 0.1 M tricine as the cathode (inner) buffer. The gel was run initially at 50 mV until the dye front had passed through into the
separating gel after which the voltage was increased to 100 mV. Once the dye front had eluted from the bottom of the separating gel the gel was stained and destained as described for SDS-PAGE gels above.
REFERENCES


References


233


References


References


References


References


References


References


References


References


References


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