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Abstract

P450s are one of the largest superfamilies of enzymes known, and have been found in almost every organism that has been investigated on the planet. The reason they are almost ubiquitous in life is because they are involved in both key biosynthetic pathways and also metabolic processes. In humans, P450s are required for the synthesis of hormones and also the metabolism of drugs. The most important of these drug metabolising enzymes is P450 3A4, which can metabolise over 60% of the drugs in use today.

This study demonstrates that highly pure P450 3A4 can be prepared utilising either of the detergents Emulgen 911 (E911) or CHAPS as the solubilising agent, and that CHAPS, a steroidal detergent, binds to P450 3A4 but is less inhibitory than E911, most likely because of its low affinity. In addition this work highlights, for the first time, that CHAPS-solubilised P450 3A4 is not a homogeneous preparation, and is in fact a mixture of oligomers, the size of the oligomeric state being affected by the amount of detergent used to purify the enzyme. Despite this aggregation, however, the preparations of P450 3A4 were shown to be active with respect to binding carbon monoxide in the reduced form, oxidising a profluorescent substrate (7-BQ), and producing an optical spin state change upon substrate binding.

Optical binding studies have suggested a correlation between a compound's affinity for P450 3A4 and its solubility, i.e. the more soluble the substrate the weaker it will bind to this protein, as has been suggested in the past. These studies have also indicated that, (i) the affinity of a substrate may be enhanced by the presence of a nitrogen that can coordinate to the iron, (ii) the active site is unlikely to be merely a large cavity where substrates can bind, (iii) the cooperative behaviour of testosterone and amitriptyline with P450 3A4 is dependent upon the buffer conditions used and (iv) one or more substrate molecules may be interacting with this protein at once and at distinct binding sites, although the nature of these sites cannot be determined.

In addition, NMR relaxation studies were carried out in an attempt to determine haem iron to substrate proton distances for P450 3A4. However, it was not possible to achieve this because the systems under investigation were all in slow/intermediate exchange on the NMR time scale, possibly because the protein samples were aggregated.
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CHAPTER ONE

Introduction
Introduction

1.1 Introduction to the cytochrome P450 superfamily

During the late 1950s a pigment was discovered in rat liver microsomes that had an unusual absorbance at 450nm when reduced with sodium dithionite and combined with carbon monoxide (Klingenberg, 1958; Omura and Sato, 1964a; Omura and Sato, 1964b). Omura and Sato identified this pigment as a haem protein and as a result of this, and its unusual spectral properties, named it “cytochrome P450” (P450s) (Omura and Sato, 1964b).

Since their discovery, P450s have been found in almost every species that has been investigated on the planet, and comprise one of the largest superfamilies of enzymes known (Anzenbacher and Anzenbacherova, 2001; Graham and Peterson, 1999; Groves and Han, 1995). The size of this enzyme family is illustrated by the fact that as of May 16, 2002 there were 977 animal, 607 plant, 190 lower eukaryote and 151 bacterial sequences named, which is a total of 1925 different sequences. However, this number does not take into account those that have not been named yet, such as about 300 in the rice genome or 150 in the white rot fungus genome. An extensive amount of sequence data and other useful information on P450s can be found at http://drnelson.utmemb.edu/CytochromeP450.html.

Cytochrome P450s are capable of regio- and stereospecifically inserting an atom of oxygen into an unactivated carbon hydrogen bond (Werck-Reichhart and Feyereisen, 2000), which is a feat that is impossible using conventional organic synthesis methods at room temperature and pressure. Indeed, even with synthetic metalloporphyrin mimic systems, comparable rates of regio- and stereoselective monooxygen insertion into an unactivated carbon hydrogen bond cannot be reached (Breslow et al., 1997). Due to their ability to monooxygenate P450s can catalyse a whole range of reactions, including hydrocarbon hydroxylation, alkene epoxidation, heteroatom oxidation, N, O, S dealkylation and many others (Sono et al., 1996). As
a result many organisms have utilised these catalysts in biologically significant reactions.

Since there are so many P450s carrying out so many varied functions, the study of the P450 superfamily has become a multidisciplinary effort, including agrochemists, organic chemists, biochemists, pharmacologists, toxicologists and many others. Some of the main goals of P450 research include the prediction of drug metabolism and the prevention of adverse drug reactions, the development of methods to improve the solubility of these enzymes and thus be able to generate crystals of membrane-bound proteins, and to engineer these enzymes for a specific function (Werck-Reichhart and Feyereisen, 2000).

### 1.1.1 Nomenclature and classification of P450s

Originally, P450s were assigned trivial names based on the sources from which they were obtained or on spectral properties, electrophoretic mobility, substrates or inducers, or in some instances numbers or letters were assigned in series (Gonzalez, 1990; Coon et al., 1992). As knowledge of their amino acid sequences developed a general nomenclature based on sequence similarity was devised (Nelson et al., 1996). Cytochrome P450 is abbreviated to CYP (or P450) and this is followed by a number for the families (those proteins with 40% or greater sequence identity), a capital letter for subfamilies (those with greater than 55% identity), and a number for individual genes, e.g. CYP2D6. These individual genes must differ by more than 3% before a different number represents them. This nomenclature has simplified the naming of P450s greatly e.g. CYP2E1 used to have many different names including: LM3a, Mkjl, Hj, alcohol oxygenase, aniline hydroxylase, N-nitrosodimethylamine demethylase, etc. Finally, particular family numbers have been reserved for certain kingdoms: 1-49 are for animals, 51-69 are for yeast, fungi and mould, 71-99 are for plants and 101+ are for bacteria. However, in the case of plant P450s far more have been identified than were initially expected and so the numbering has continued from 700 (http://drnelson.utmem.edu/biblioD.html).

Once alleles of the human P450 genes were isolated and sequenced an extension of this nomenclature system was devised in order to take these genes into account (Daly
et al., 1996; Antonarakis, 1998; den Dunnen and Antonarakis, 2001). In brief, the
gene and allele is separated by an asterisk followed by Arabic numerals and upper-
case Roman letters with less than four characters to name the allele, e.g. CYP1A1*3,
CYP1B1*22, CYP2D6*10B. If there are extra copies (n) of a gene placed in tandem
the entire allelic arrangement will be referred e.g. CYP2D6*2Xn. The wild type
allele is defined as *1 (or *1A and *1B in the case where the sequences differ only
slightly).

In order for P450s to carry out their monooxygenation reactions they require two
electrons, which they normally obtain from either reduced nicotinamide adenine
dinucleotide (NADH) or the phosphate-containing analogue (NADPH), usually via
separate intermediate electron transfer proteins. P450s have been classified
depending upon how electrons from NAD(P)H are delivered to the catalytic site
(Werck-Reichhart and Feyereisen, 2000). Class I proteins need two electron-transfer
proteins, a flavin adenine dinucleotide (FAD)-containing cytochrome P450 reductase
(CPR) and an iron sulphur redoxin; bacterial and eukaryotic mitochondrial P450s fit
into this category e.g. P450 101, 107 and 108. Class II proteins are dependent on
only one electron-transfer protein, which is an FAD/flavin mononucleotide (FMN)-
containing CPR; most other eukaryotic P450s belong to this type e.g. P450 2D6 and
3A4. A notable exception to this generalisation is the bacterial fatty acid
hydroxylase P450 102 (Narhi and Fulco, 1986), obtained from Bacillus megaterium,
which unlike other P450s is fused to its (FAD- and FMN-containing) electron-
transfer partner, and thus resembles a class II P450. Class III proteins are self
sufficient, since they do not require an external source of oxygen or electrons. These
P450s actually catalyse a rearrangement of an organic compound rather than a
monooxygenation reaction. Examples include prostacyclin synthase and
thromboxane synthase (Hecker and Ullrich, 1989), which act as isomerases. Class
IV P450s, such as P450 55A1, are very rare and can utilize NAD(P)H directly
(Mansuy and Renaud, 1995). P450 55A1 has been found in a fungus, Fusarium
oxysporum. It is a soluble protein that utilises NADH to catalyse the reduction of
nitrate or nitrite, generated by denitrification, to nitric oxide (N₂O). Interestingly, the
reduction of the P450 haem iron can only occur once the nitrate or nitrite has
coordinated to it. Once this has occurred, the haem iron can transfer an electron to
either of these molecules and generate N₂O. However, the mechanism by which N₂O

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is formed is still unclear. However, more recently P450s have been found that do not fit into this classification system, e.g. P450 51FX, which is naturally fused to a ferrodoxin domain at the C-terminus via an alanine-rich linker (Jackson et al., 2002).

1.1.2 Occurrence, localisation and function

Typically, bacterial genomes only have a few P450 genes but notable exceptions are *Escherichia coli* that has none, and *Mycobacterium tuberculosis* that has twenty (Directory of P450 containing systems at http://www.iceb.trieste.it/~p450srv/). These prokaryote P450s are soluble proteins, which has assisted in their purification, and in some cases their crystallisation (see section 1.1.3). *M. tuberculosis* has the largest number of P450 genes yet found in a single bacterial genome, and it is thought that some of the drug resistance shown by this bacterium could be due to it recruiting these P450 genes for the purpose of oxidising these compounds (Nelson, 1999). Normally, the P450s present in bacteria are used to biodegrade organic compounds for metabolic energy, e.g. P450 101, which allows *Pseudomonas putida* to utilise camphor (Martinis et al., 1991), and P450 108, which allows a *Pseudomonad* to exist on α-terpineol (Hasemann et al., 1994). However, some bacterial P450s are involved in biosynthetic processes. For example, P450 107, which was found in *Saccharopolyspora erythaea*, is involved in the biosynthesis of erythromycin.

Lower eukaryotes genomes also have only a few P450s; for example, the two fungal species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have only three and two, respectively (Nelson, 1999). In higher eukaryote genomes, however, the number of P450 genes increases dramatically, for example, the fruit fly *Drosophilia melangaster* has 83 (The P450 site at the University of Arizona at http://www.ag.arizona.edu/p450/), the nematode *Caenorhabditis elegans* has 80, and humans have 55 (Werck-Reichhart and Feyereisen, 2000). The organisms with the largest number of P450 genes are higher plants such as *Arabidopsis thaliana*, which has 286. Generally, the higher eukaryotic P450s are membrane bound. Class I eukaryotic P450s are bound to the inner membrane of the mitochondria and are involved in the biosynthesis of steroid hormones and vitamin D₃ in mammals (Anzenbacher and Anzenbacherova, 2001). These mitochondrial P450s are also
present in insects and nematodes, but at the present time none have been found in plants. Class II P450s are the most common in eukaryotes, and are found on the outer face of the endoplasmic reticulum. The NADPH-cytochrome P450 reductase (CPR) partners are normally separate proteins that are also associated with this membrane via their amino-terminal hydrophobic anchors. In addition to these CPRs, cytochrome \( b_5 \) is involved in the transfer of electrons to P450, and has been found to enhance the activity of some of these enzymes, especially P450 3A4 (see section 1.3.1). In fungi the functions of P450s include metabolism of lipid carbon sources, synthesis of membrane sterols and mycotoxins, and detoxification of phytoalexins. In animals, they are required for the biosynthesis of signalling molecules, oxylipins and steroid hormones (Hasler et al., 1999; Guengerich, 1995). For example, in humans P450 11A1 catalyses the side chain cleavage of cholesterol into pregnenolone, which is the precursor for the synthesis of mineralocorticoids, glucocorticoids, progestins and sex hormones. In order to then produce the female sex hormone estradiol P450 17 and 19A1 are also required (Kagawa and Waterman, 1995). Reduced activity in P450 11A1 can lead to hypertension, feminisation, and glucocorticoid insufficiency (Guengerich, 1995). In plants, P450s are involved in the synthesis of hormones, cutins and in many of the pathways of secondary metabolism, which include lignification and the production of flower pigments and defense chemicals (Kahn and Durst, 2000).

Class II P450s, from all organisms, are required for the catabolism of xenobiotics, which occasionally leads to the generation of carcinogenic products. For example, the fact that human P450 1A1 is capable of activating polycyclic aromatic hydrocarbons, which are present in cigarette smoke, and that it is mainly expressed in the lungs suggests that this P450 may be involved in lung cancer (Alexandrov et al., 1992; Guengerich, 1995; Kouri et al., 1982; McLemore et al., 1990). In fact, a number of reports have indicated that most of the chemical carcinogens in the environment are not active enough in themselves to interact with intracellular DNA, but induce tumors only after metabolic activation by a variety of drug-metabolising enzymes including P450 (Shimada et al., 1997). The major P450s involved in the activation of most procarcinogens and promutagens have been reported to be P450s 1A1, 1A2, 2E1 and 3A4 (Guengerich and Shimada, 1991; Gonzalez and Gelboin, 1994).
Fortunately though, catabolic P450s normally convert toxic compounds into more polar ones that are easier to excrete, either directly or after conjugation with water-soluble components such as glucuronic acid or glutathione (Porter and Coon, 1991). Some organisms have adapted so that they are able to metabolise a particular toxin in their food in order to give them a competitive advantage. An example of this is that only certain *Papilio* butterfly larvae can feed on toxic furanocoumarin-containing plants because they are able to produce P450 6B1 (Wilson, 2001). Thus, these catabolic P450s make it possible for an organism to survive the toxins that are produced by others as deterrents (Werck-Reichhart and Feyereisen, 2000; Anzenbacher and Anzenbacherova, 2001). Often the genes for the expression of these catabolic P450s are induced by the presence of exogenous chemicals (Schuetz, 2001; Whitlock and Denison, 1995), which is the case in human P450 3A4 (see section 1.3.1).

The fact that there are a greater number of P450s present in higher eukaryotes could be a direct result of plant-animal co-evolution; as plants generate biosynthetic pathways to new toxic secondary metabolites, animals must develop methods to detoxify these compounds so that they can still consume them (Gonzalez and Nebert, 1990).

### 1.1.3 Structural characteristics and substrate binding

The first crystal structure to be reported was in 1986 for P450 101 (Poulos et al., 1986). Since then many other bacterial P450s have been crystallised, e.g. P450 102 (Ravichandran et al., 1993), 108 (Hasemann et al., 1994), 107 (Cupp-Vickery and Poulos, 1995), 55A1 (Park et al., 1997), 119 (Yano et al., 2000), 51 (Podust et al., 2001), and 175A1 (Yano et al., 2003). Although the sequence identity between these P450s is quite low, which probably reflects the fact that they bind very different types of molecule, the crystal structures demonstrate that their general topology and structural fold is conserved (Graham and Peterson, 1999), see also Figure 1-1, and thus it is believed that the majority of P450s will fold in the same way. From these structures it has been possible to determine some key structural features about these proteins (Figure 1-2).
Figure 1-1: Comparison of schematic diagrams for P450 101 (I), 102 (haem domain) (II), 107 (III) and 108 (IV), based on their crystal structures.

Diagrams obtained from http://www.icgeb.trieste.it/p450.
The greatest structural conservation is at the core of the protein around the haem and suggests that P450s bind haem in a similar way; also it could possibly indicate a common mechanism of oxygen activation by electron and proton transfer. The conserved core of the protein contains a four-helix bundle (D, E, I, and L) and the helices J and K. Helix L is on the proximal face of the haem and contains the haem-binding loop. Other structurally conserved elements are β-sheet 1, containing five strands, and β-sheet 2, containing two strands, which help form the hydrophobic access channel.
The haem-binding loop contains the most characteristic P450 consensus sequence (Phe-X-X-Gly-X-X-X-Cys) (Gotoh and Fujii-Kuriyama, 1989) with the absolutely conserved cysteine that serves as the fifth ligand of the haem iron. The cysteine is the reason for the characteristic 450nm absorbance found in the reduced carbon monoxide bound form, and is thought to help stabilise the highly reactive intermediate formed once the oxygen bond is cleaved, see section 1.1.4 (Sono et al., 1996). Helix K is also on the proximal side of the haem and contains the absolutely conserved Glu-X-X-Arg sequence, which is thought to be involved in stabilising the core structure. Finally the central part of helix I, which is on the distal side of the haem, has another P450 signature sequence (Ala/Gly-Gly-X-Asp/Thr-Thr/Ser) that is required for proton transfer (Werck-Reichhart and Feyereisen, 2000). However, this signature sequence is not present in all P450s, e.g. class III P450s, perhaps reflecting the fact that these proteins catalyse a rearrangement rather than an oxidation reaction (Mansuy and Renaud, 1995).

Nonconserved variable regions are usually connected with substrate or CPR partner binding. Gotoh (Gotoh, 1992) was able to identify regions that were associated with substrate recognition by comparing the sequences of the P450 2 family and P450 101. These variable regions were assigned SRS (Substrate Recognition Site) numbers, which are now the start points for site-directed mutagenesis on any new P450 under investigation (Domanski and Halpert, 2001). From this work, helices A, B, B', F and G, and their adjacent loops, are believed to be involved with substrate binding. Loops B-B' and B'-C are present in the active site (SRS-1), and helices F and G, and the F-G loop, generate part of the access channel and the upper surface of the active site (SRS-2 and SRS-3). The centre part of helix I (SRS-4) is also involved in substrate binding but, because of its previously mentioned role in the reaction mechanism, it is highly conserved, unlike the rest of these regions. Finally, both the region at the N terminus of β-strand 1-4 (SRS-5) and the β-turn at the end of β-sheet 4 protrude into the active site, and thus are also implicated in substrate binding.

Substrate binding to the active site of P450s requires diffusion into the protein's interior, where the haem moiety is located. The buried nature of the haem in P450s was first demonstrated by the crystal structure of substrate-free P450 101 (Poulos et
al, 1986). The substrate bound crystal structure of P450 101 (Poulos et al, 1987) revealed that camphor binds to the active site without any clear channel to the aqueous medium, which suggested that the protein must undergo a fairly large reorganisation before the substrate can gain access. Two regions of the protein were found to shift orientation significantly when an inhibitor, with a long aliphatic chain, was bound to the protein; these were the B' helix and the loop joining the helices F and G (Raag et al., 1993). In addition, measurements of substrate dissociation constants at different ionic strengths suggested that an Asp-Lys salt bridge, which seemed likely from the crystal structure (Poulos et al, 1987), might also be involved in substrate binding (Deprez et al., 1994). Although the salt bridge is particular to P450 101, the involvement of the B' helix and the F-G loop in substrate binding does seem to be more general (von Wachenfeldt and Johnson, 1995).

P450 101 is a good example of a substrate specific P450 because once camphor is in the active site, complementary hydrophobic contacts and a hydrogen bond hold it in the correct orientation to be hydroxylated only at the 5-exo position (Martinis et al., 1991). As a result of these specific binding interactions camphor binds quite tightly, with a dissociation constant ($K_d$) of 1.6 $\mu$M (Atkins and Sligar, 1988), and any alterations to the general shape or properties of this compound result in an increase in the $K_d$. For example, norcamphor, which is only different to camphor by three methyl groups (Figure 1-3), only binds with a $K_d$ of 150 $\mu$M (Mueller et al., 1995).

![Camphor and Norcamphor](image)

**Figure 1-3: Comparison of camphor and norcamphor**

The $K_d$ values reported for camphor (Atkins and Sligar, 1988) and norcamphor (Mueller et al, 1995) are for binding to P450 101.
In contrast, P450s that are involved in detoxification processes are capable of accommodating a very broad range of substrates (Porter and Coon, 1991); the best example of this is P450 3A4 (see section 1.3). Therefore, P450 101 and other substrate specific P450s may not be the best models for a detoxification enzyme such as P450 3A4.

Until recently it could only be assumed that membrane bound eukaryotic P450s would fold in the same way as their soluble prokaryotic counterparts. However, this assumption has become significantly more valid since the crystal structure of rabbit P450 2C5 became available in 2000, which showed that this eukaryotic P450 had a very similar fold to the bacterial P450s (Figure 1-4a).

It should, however, be noted that this protein was not native P450 2C5. In actual fact Williams et al (Cosme and Johnson, 2000; Williams et al., 2000) had to delete 22 residues from the N-terminus, include a histidine tag on the C-terminus, and make five substitutions in the FG region, derived from 2C3, before the protein was soluble and monomeric i.e. in a state ready for crystallisation. Interestingly, this work highlighted how the FG region was intimately involved in self-aggregation and thus led to the postulation that this region may be involved in membrane binding (Cosme and Johnson, 2000) (Figure 1-4b) as had been suggested by Graham-Lorence and Peterson (Graham-Lorence and Peterson, 1995). Also it was found that these changes to the FG region either did not alter or enhanced the rates of progesterone (methyl group-21) hydroxylation (Cosme and Johnson, 2000), which suggested that this crystal structure should not be significantly different to wild type P450 2C5.

When the structure of P450 2C5 was compared to P450 102, the bacterial enzyme with the greatest sequence identity, although it was found to possess all the same structural elements that define the active site their spatial arrangement with respect to one another was very different (Williams et al., 2000). The greatest difference between the structures was found to be the arrangement of the mostly β-sheet N-terminal domain with respect to the α-helical haem core domain. In actual fact the bacterial P450 with the closest overall structure to P450 2C5 was found to be P450 108. Also, when the relative positions of the SRSs were compared between P450
**Figure 1-4: Ribbon representations of P450 2C5.**

a) Comparison of the overall fold of P450 101 (I) and P450 2C5 (II).

b) The following key structural elements are highlighted on this representation of P450 2C5: the iron (purple) and the haem (green), which are essential for catalytic activity, the helices I, F and G (blue), which are involved in substrate binding, and the F-G loop and N-terminus (red), which are believed to be involved in the association of the P450 to the endoplasmic reticulum (transparent yellow rectangle labelled membrane). Part a (II) and b were produced using Accelrys ViewerLite 4.2.
2C5 and 102 it was found that only the position of SRS 4 was highly conserved, which is not surprising since this is part of the I helix. The remainder of the SRSs were at divergent positions when compared to P450 102, most likely reflecting the fact that this bacterial P450 oxidises long thin fatty acid chains while P450 2C5 oxidises bulky steroids.

1.1.4 Catalytic cycle

The catalytic cycle of P450s has been elucidated mainly through the work carried out on P450 101 (Mueller et al., 1995) (Figure 1-5).

The P450 catalytic cycle begins with the enzyme in a six-coordinate low spin ferric state (Fe$^{3+}$), 1, with an exchangeable water ligand trans to the proximal cysteinate. Substrate binding causes the water ligand to dissociate, thus generating a five-coordinate high spin ferric state, 2. In the case of P450 101, this conversion of the ferric iron from low to high spin results in a significant increase in the redox potential of the enzyme haem (Mueller et al., 1995), thus allowing electron transfer from reduced putidaredoxin to form the five-coordinate high spin ferrous state (Fe$^{2+}$), 3.

This requirement for a substrate to bind before the first electron reduction to occur is believed to be a physiological gate to the formation of the reactive oxygen intermediate within P450 101, since otherwise this intermediate and its breakdown products could damage the activity of this protein, in addition it represents a waste of energy that could be used for another biological purpose. Although this physiological gate is also present in P450 102 (Narhi and Fulco, 1986), this is not the general case for other P450s (Guengerich and Johnson, 1997). For example, Guengerich and Johnson (Guengerich and Johnson, 1997) demonstrated that P450 1A2 was reduced rapidly and this rate was unaffected by the presence or absence of substrate, whereas P450 2C9 was reduced very slowly in the absence of substrate but this rate could be enhanced by the addition of (S)-warfarin or tolbutamide. They (Guengerich and Johnson, 1997) also found that P450 3A4 was reduced at highly variable rates depending on what system was employed e.g. a P450 3A4:NADPH-
Chapter One

Figure 1-5: Catalytic cycle of P450 101.

Each structure within the catalytic cycle illustrates the oxidation state and coordination geometry of the iron within the haem of P450 101. Note that, although substrate R-H is only depicted in states 2 and 6, it is actually present in states 3, 4, 5 and 7 as well. This figure was mainly adapted from Sono et al., 1996.
CPR fusion protein in the presence of cytochrome $b_5$ and testosterone was reduced at 9-12 min$^{-1}$, whereas P450 3A4 expressed in baculovirus membranes in the presence of testosterone was reduced at 2300 min$^{-1}$.

Once 3 is formed, dioxygen can bind to form the oxyferrous state, 4. Alternatively, carbon monoxide can bind at this stage to form the ferrous-CO inhibitor adduct, 7, which has a characteristic absorbance at 450nm. Addition of the second electron to 4, normally the rate-limiting step of the reaction (Sono et al., 1996), is believed to generate a ferric peroxide adduct, 5a, which can then be protonated to give a hydroperoxide complex, 5b. Finally, heterolytic cleavage of the O-O bond, which is achieved by specifically protonating the same oxygen atom again to form water, results in the production of the proposed oxo-ferryl porphyrin radical intermediate, 6, that actually carries out the monooxygenation reaction. However, there is some evidence to suggest that species 5a and 5b are alternative oxidants for some P450 mediated reactions (Akhtar et al., 1982; Vaz et al., 1996; Mansuy, 1998). The P450 catalytic cycle is completed when 6 inserts an oxygen atom into the R-H bond to form the alcohol product and 1. This insertion step is believed to take place by 6 abstracting a proton from the substrate to form a hydroxyl radical, which can then recombine with the substrate radical to yield the alcohol product. In the case of a substrate specific P450, such as P450 101, this reaction usually proceeds with 100% coupling of electron transfer to product. However, when the P450 is less specific, e.g. P450s involved with drug metabolism, uncoupling occurs that results in the production of superoxide from 4 and peroxide from 5a or 5b. In addition, state 6 can produce water through a 4-electron uncoupling reaction (Sono et al., 1996).

Evidence for the structures of these various states in the catalytic cycle have been derived using a number of different methods. Initially, various spectroscopic techniques were used to elucidate the nature of states 1-4 and 7 (Sono et al., 1996) and references therein. Later, however, crystals of P450 101 in states 1 (Poulos et al., 1986), 2 (Poulos et al., 1987), 3, 4, 6 (Schlichting et al., 2000), and 7 (Raag and Poulos, 1989) were obtained, thus confirming these proposed states, although Schlichting et al (Schlichting et al., 2000) were slightly cautious about their crystal structure data of state 6. However, such a high valent iron-oxo species does already exist for peroxidase enzymes, which is called compound I (Sono et al., 1996).
addition, the presence of a ferryl iron species was detected using Mossbauer spectroscopy when the activated oxygen intermediate of P450 101 was generated in the absence of substrate (Schunemann et al., 2002). Although no direct evidence exists for the presence of intermediates 5a and 5b, some chemical reactivities of P450 that are consistent with such species have been demonstrated (Akhtar et al., 1982; Vaz et al., 1996; Mansuy, 1998).

Interestingly, it has been found that various oxygen atom donors, such as iodosobenzene and cumene hydroperoxide, can provide the two electrons and O₂ that are required for the normal catalytic cycle. These compounds can react directly with 2 via what is called the peroxide shunt mechanism to form an active oxygen intermediate, which is assumed to be similar to 6, which can then oxidise substrates (Hrycay et al, 1976).

1.2 Drug metabolism in humans

The main role of P450 3A4 is to metabolise drugs in humans and therefore it important to understand how the body deals with these xenobiotics compounds.

The metabolism of drugs in humans has been categorised into two phases (Manahan, 1989):

Phase I modification
Phase II conjugation

Phase I metabolism results in the functionalisation of the drug so that it can either be excreted directly or conjugated in phase II. This step is mainly carried out by P450s, but other enzymes such as reductases and hydrolases participate as well. The next phase involves conjugating the modified drug with polar groups, thus making it even more water soluble and so easier to excrete. Proteins that participate in this step are uridine diphosphate glucuronyltransferases, glutathione transferases, N-acetyl transferases and sulphotransferases, which add glucuronide, glutathione, acetyl and sulphate groups, respectively, to the drug under metabolism.
Once the drug has been chemically modified the body can excrete it in a number of different ways, for example, in urine.

1.2.1 P450s involved in drug metabolism

In humans, the P450s involved in metabolism are mainly found in the liver, but they are also expressed in other tissues such as the brain, skin, lungs and heart (Guengerich, 1995; Werck-Reichhart and Feyereisen, 2000). Since there is a considerable amount of microsomal P450 in a human liver, e.g. approximately 7500 nmoles in a 1.5-kg liver (Guengerich, 1990), it is clear that the body continually has to metabolise an enormous number of endogenous and xenobiotic compounds.

Cytochrome P450s 1A2, 2C9, 2C19, 2D6, 2E1 and 3A4 are considered to be the main P450s involved in drug metabolism (Shapiro and Shear, 2002). Table 1-1 summarises the relevant information on these P450s. From Table 1-1 it can be seen that these main P450s are quite complementary towards one another. P450 3A4 metabolises the majority of lipophilic substances. However, if the substrate is charged then P450s 2C9, 2C19 or 2D6 can be utilised instead. Finally, if the xenobiotic has aromatic character it can be metabolised by P450 1A2 and if it is small and soluble it can be dealt with by P450 2E1. Thus, together these six P450s can metabolise just about all types of compound that might be absorbed by the body.

1.2.2 Factors that determine P450 activity

P450 activity differs from one person to another. The main reason for this is genetic polymorphism of these proteins, which causes different individuals to express different amounts and/or different alleles of these P450s. As a result, three main phenotypes have been described for various P450:

1) Poor metabolisers. Individuals with genetically determined low levels of activity.

2) Extensive metabolisers. Individuals who have normal activity.
### Table 1-1: Tissue localisation and typical substrates metabolised by the human drug metabolising P450s (reproduced from Anzenbacher and Anzenbacherova, 2001).

<table>
<thead>
<tr>
<th>P450</th>
<th>Localisation</th>
<th>Typical substrate(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Liver</td>
<td>Polycyclic aromatic hydrocarbons, aromatic amines, caffeine</td>
</tr>
<tr>
<td>2C9</td>
<td>Liver</td>
<td>Usually are weakly anionic and fairly lipophilic, <em>e.g.</em> tolbutamide, diclofenac.</td>
</tr>
<tr>
<td>2C19</td>
<td>liver, heart</td>
<td>Usually are neutral or weakly basic and moderately lipophilic, <em>e.g.</em> (S)-mephenytoin, omeprazole, diazepam</td>
</tr>
<tr>
<td>2D6</td>
<td>liver, brain, heart</td>
<td>Varied structures, though usually have basic nitrogen, <em>e.g.</em> amitriptyline, codeine, debrisoquine.</td>
</tr>
<tr>
<td>2E1</td>
<td>liver, lung, brain, endothelium, placenta, lymphocytes</td>
<td>ethanol, nitrosoamines, acetaminophen</td>
</tr>
<tr>
<td>3A4</td>
<td>liver, gastrointestinal tract, kidney, lung, brain, endothelium, placenta, lymphocytes</td>
<td>Extremely varied structure but often highly lipophilic, <em>e.g.</em> cyclosporin, testosterone, and Taxol. (see also section 1.3).</td>
</tr>
</tbody>
</table>

### Table 1-2: Effect of P450 activity on therapeutic drug effects.

<table>
<thead>
<tr>
<th>Drug activity</th>
<th>Phenotype</th>
<th>Therapeutic effect</th>
<th>Possible Toxic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>Poor</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Extensive</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Ultra-rapid</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Pro-Drug</td>
<td>Poor</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Extensive</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Ultra-rapid</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Table 1-2: Effect of P450 activity on therapeutic drug effects.*
3) Ultra-rapid metabolisers. Individuals who have more than the normal complement of functional genes and/or a P450 allele with higher than normal activity.

Since the P450s in families 1-3 are responsible for 70-80% of all phase I dependent metabolism of drugs (Ingelman-Sundberg, 2002) it should be possible to predict an individual's response to a drug, assuming that their phenotype is known for the P450 involved in the metabolism of that drug. For example, a poor metaboliser may have a greater response to a drug than an extensive metaboliser. In addition, though, the poor metaboliser could have a greater risk of experiencing toxic effects from the same drug than the extensive metaboliser, if the drug is highly dependent on clearance to an inactive metabolite by that particular P450. Alternatively, a drug might not affect an ultra-rapid metaboliser at all because therapeutically active plasma concentrations cannot be reached.

A further complicating factor to determining the effect of a drug on an individual is that some drugs, called pro-drugs, are only active after metabolic modification, often by P450s. Thus, therapeutic and/or possible toxic effects can occur depending upon the P450 phenotype of the individual and on whether the drug or its P450-generated metabolite is active. These results are summarised in Table 1-2.

Although all of the main drug metabolising P450s exhibit some degree of genetic polymorphism, the most clinically important polymorphisms are seen with P450 2C9, 2C19 and 2D6 (Ingelman-Sundberg, 2002; Wrighton and Stevens, 1992; Anzenbacher and Anzenbacherova, 2001; Shapiro and Shear, 2002; Rodrigues and Rushmore, 2002).

For example, P450 2D6 produces the three main phenotypes described above. The poor metaboliser arises (Rodrigues and Rushmore, 2002) through alleles CYP2D6*4 (Splicing defect causing the expression of inactive protein), CYP2D6*5 (Gene deletion causing no enzyme expressed), and CYP2D6*10 and CYP2D6*17, which express mutated forms of this protein that are either unstable or have reduced activity in comparison to the wild type. The ultra-rapid metaboliser arises because of allele CYP2D6*2xn, which is a gene duplication (Rodrigues and Rushmore, 2002).
Studies have shown that ethnic differences exist with respect to the number of individuals in a population who are poor, extensive or ultra-rapid metabolisers, e.g. 7% of the Caucasian population have defective CYP2D6 genes, whereas in the Asian population there are nearly 50% (Anzenbacher and Anzenbacherova, 2001). The P450 2D6 poor metaboliser phenotype has been associated with Parkinson's disease (McCann et al., 1997). However, it has also been reported that this phenotype could protect against urinary bladder cancer (Abdel-Rahman et al., 1997) and possibly the development of lung carcinoma (Caporaso et al., 1990). Low P450 2D6 activity may also be important in drug metabolism, since elevated levels of tricyclic antidepressants could lead to cardiotoxic effects, and with antiarrythmics life threatening arrhythmias may develop (Ingelman-Sundberg et al., 1999).

In addition to the genetic factors that determine the basal P450 activity an individual possesses, a particular P450's activity can be modulated by induction and/or inhibition.

1.2.2.1 Induction

Induction of a P450 occurs when a substance, known as the inducer, accumulates above a critical concentration in the cell. Since the resultant effect of induction is to increase expression of a particular P450, its overall activity will increase. For some P450s the inducer is also the substrate, such as polyaromatic hydrocarbons for P450 1A1 and ethanol for 2E1, thus demonstrating how some of these proteins have evolved to regulate the amounts of particularly toxic compounds (Whitlock and Denison, 1995; Shapiro and Shear, 2002). However, sometimes induction leads to enhanced chemical toxicity. For example, high concentrations of acetaminophen result in the saturation of detoxification pathways, which leads to reactions mediated by P450s that generate reactive electrophiles, which bind to cellular macromolecules and cause hepatic necrosis. Thus cytochrome P450 induction would increase the severity of acetaminophen toxicity by increasing the production of the electrophilic metabolites (Whitlock and Denison, 1995).
1.2.2.2 Inhibition

The inhibition of P450s can lead to an increase in plasma drug concentrations, increased drug response and toxic effects. Alternatively, P450 inhibitors can be coprescribed with drugs which would normally be metabolised very quickly by a particular P450, so that the drug has a chance to elicit its effect. Thus it is extremely important to know what compounds inhibit what P450s and how they achieve this effect (Shapiro and Shear, 2002).

P450s are susceptible to inhibition at various stages throughout the catalytic cycle, including substrate binding, transfer of the first or second electron, and binding of molecular oxygen subsequent to the first electron transfer (Ortiz de Montellano and Correia, 1995). An example of an inhibitor that competes with substrates for the active site of most P450s is ketoconazole (Shapiro and Shear, 2002), however, it is highly selective for P450 3A4 as indicated by its low \( K_i \) value of 0.015-8 \( \mu \)M (Thummel and Wilkinson, 1998). This compound is an effective inhibitor because it is able to coordinate directly to the haem iron and thus as well as blocking substrate access it prevents the formation of the oxyferrous species. Interestingly, there are also competitive inhibitors present in our diet. For example, grape fruit juice is able to inhibit P450 3A4 (Anzenbacher and Anzenbacherova, 2001).

Some inhibitors, however, are non-competitive. These inhibitors are often oxidised to form a species that covalently binds to either the protein or the haem group. Examples of such mechanism-based inhibitors include terminal alkenes or alkynes, dihydropyridines, dihydroquinolines and alkyl hydrazines (Ortiz de Montellano and Correia, 1995).

1.3 Cytochrome P450 3A4

1.3.1 Introduction

Of the several drug metabolism P450s described above, the P450 3A subfamily have the greatest importance, since collectively they are by far the most abundant of all the human P450 isoforms, their substrate specificity is extremely broad (which can lead
to undesirable drug-drug interactions) and their catalytic activity is affected by numerous factors (Thummel and Wilkinson, 1998).

### 1.3.2 Tissue localisation

P450 3A4 represents the major isoform of the human P450 3A subfamily, although the term P450 3A4 is generally taken to indicate a collective contribution of both this isoform and P450 3A3, which has >98% cDNA sequence similarity (Thummel and Wilkinson, 1998). P450 3A4 is universally found to be the dominant P450 in adult human liver where on average it constitutes about 30% of total P450 protein, but this can be increased to nearly 60% upon induction by steroids and barbiturates (Thummel and Wilkinson, 1998; Anzenbacher and Anzenbacherova, 2001). This protein is also present in relatively high amounts in the small intestinal epithelium, about 50% of hepatic levels and 70% of total P450 protein. Finally, P450 3A4 has also been found in the kidney. However, it is only present in about 30% of renal tissue samples and the mechanism for such polymorphic expression is not clear (Thummel and Wilkinson, 1998).

Two other isoforms that have distinct sequences exist in the human P450 3A subfamily, which are P450s 3A5 and 3A7 (Nelson et al., 1996), although they do have >85% sequence identity with P450 3A4 (Gillam et al., 1997). P450 3A5 is only present in 10-30% of adult human livers and then at only 10-30% of P450 3A4 levels (Wrighton et al., 1989). P450 3A7 is mainly expressed in fetal liver (Komori et al., 1990), from which it was first isolated and characterised (Kitada and Kamataki, 1979), however, it may also be selectively expressed in adult livers at lower levels than P450s 3A4 and 3A5 (Thummel and Wilkinson, 1998).

### 1.3.3 Studying P450 3A4 in vivo

Normally, P450 isoforms that demonstrate broad interindividual variability and/or significant alteration in their catalytic activity by environmental factors are characterised by phenotyping using an appropriate *in vivo* probe, *e.g.* P450s 2C19 and 2D6. However, employing this method with P450 3A4 is difficult in practice due to the following complications (Thummel and Wilkinson, 1998):
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1) The route of administration is important since P450 3A4 can metabolise drugs where they are absorbed e.g. the gastrointestinal tract and the kidney, as well as the liver.

2) The effect of inhibitors or inducers is often concentration dependent; therefore it is highly desirable to maintain a constant concentration of these chemicals so that an accurate picture can be obtained. However, in vivo this is extremely difficult to achieve due to the system being extremely variable and dynamic.

3) P450 3A4 demonstrates a large range of interindividual variability in the level of its activity in various populations. Some studies have shown that levels of P450 3A4 activity and expression vary by up to 60-fold (Anzenbacher and Anzenbacherova, 2001). Although P450 3A4 has 19 alleles (http://www.imm.ki.se/CYPalleles/cyp3a4.htm) none of these has been shown to abolish activity, therefore it seems likely that such interindividual variability exists because of differences in the basal expression level of this enzyme (Thummel and Wilkinson, 1998).

4) An ideal and universally applicable in vivo substrate has yet to defined for P450 3A4. For example, the ratio of 6β-hydroxycortisol to cortisol in urine has been used in the past as an in vivo probe for P450 3A4 (Anzenbacher and Anzenbacherova, 2001). In several studies this was shown to be a successful probe since this urinary ratio increased when patients were treated with P450 3A4 selective inducers e.g. rifampicin (Horsman et al., 1992). However, a similar study with the P450 3A4 selective inhibitor troleandomycin produced no consistent change in cortisol 6β-hydroxylation, whereas the N-demethylation of erythromycin, another in vivo probe, was significantly affected (Watkins et al., 1992).

Thus, although in vivo studies are in principle the most relevant to understanding how a drug will be metabolised by P450 3A4, and other P450s, the results are not always easily interpreted.

1.3.4 Studying P450 3A4 in vitro

In vitro studies clearly cannot reflect the very complicated in vivo situation, however, they do have the advantage that conditions can be more closely controlled and
altered. As a result they can be used to gain an insight into the specificity and the *modi operandi* of these P450s. This data should then help the interpretation of the *in vivo* results, and thus produce a better overall understanding of the whole system.

The next level down, in complexity, from the *in vivo* situation is to use human tissue samples that contain P450 3A4. The major problem with this methodology is obtaining suitable human material (Thummel and Wilkinson, 1998). Postmortem samples cannot be used since the material must be as fresh as possible. Thus material must be obtained from willing patients. However, organs from donor patients that are unsuitable for transplantation can only be used when the cause of liver rejection does not dramatically alter *in vitro* cell viability or metabolic function. Furthermore, if the organ donors have previously received drugs that can induce P450 3A4, such as dexamethasone or barbituates, then their organs will be less desirable since they do not reflect the basal levels of P450 3A4 present in a typical human. Due to the problems of obtaining large quantities of fresh tissue alternative approaches have been investigated. One such approach is the use of human hepatocytes, since these retain all the relevant enzymes and cofactors and thus can still provide a good correlation to the *in vivo* situation (Pichard et al., 1990; Maurice et al., 1992). A major problem, however, with using human tissue samples or human hepatocytes is the fact that they still contain all the other P450s, and thus some method is required to highlight the specific function of P450 3A4. Selective inhibitors are usually used for this purpose. Peptide-based (Wang and Lu, 1997) and monoclonal (Gelboin et al., 1995) antibodies have been reported as being selective inhibitors for P450 3A4, as have chemicals such as ketaconazole and progestin gestodene (Newton et al., 1994).

Another *in vitro* approach is to heterologously express recombinant P450s and then utilise them either in the host's membrane or as reconstituted purified proteins. Although this method readily generates large quantities of just a single P450, the resultant preparation is clearly not very similar to the *in vivo* situation. However, it is only at this level of simplification that the true specificity and functionality of a particular P450 can be ascertained.
In 1986 the cDNA of P450 3A4 was cloned and the sequence determined (Beaune et al., 1986). It then became possible to express this protein in various hosts including yeast cells (Brian et al., 1990), Hep G2 cells (Shou et al., 1994), SF9 insect cells (Koley et al., 1995), COS cells (Ball et al., 1992) and E.coli (Gillam et al., 1993). In addition, P450 3A4 has been expressed in several mammalian cell lines including V79 Chinese hamster (Rauschenbach et al., 1995) and human lymphoblastoid (Crespi et al., 1991). In most of these cells an NADPH-CPR protein is also expressed and so the system is already catalytically active, but for yeast cells and E.coli the situation is more complicated. P450 3A4 does not couple well with yeast NADPH-CPR (Brian et al., 1990) and E.coli does not produce any endogenous CPR at all. However, by developing alternative constructs that also expressed human CPR as well as P450 3A4 catalytically active systems were produced (Renaud et al., 1990; Blake et al., 1996).

The final level of simplification is to purify the P450 from the membrane using detergents and then to reconstitute its catalytic activity by the addition of phospholipids, usually dilauroylphosphatidylcholine, and NADPH-CPR. In this way it is possible to have almost total control over the system under investigation and thus be able to analyse the effect on activity of every component within it. However, the P450 3A family seem to require more complicated reconstitution conditions than other P450s, (Guengerich, 1999) and reference therein. When purified P450 3A2 (from rat liver microsomes) was reconstituted with the standard phospholipid and CPR the system did not show testosterone 6β-hydroxylase activity, but by adding cytochrome b5 activity could be restored (Imaoka et al., 1988). Also, it was found that by using a phosphatidylserine:lecithin (1:2) mix and sodium cholate the rate of hydroxylation could be enhanced by twenty fold. In 1992, Imaoka et al (Imaoka et al, 1992) reported that the reconstitution of P450 3A4 activity was also sensitive to cytochrome b5, the phospholipid used and sodium cholate. Since then it has been found that the catalytic activity of P450 3A4 can be modulated by the buffer (Shet et al., 1993), monovalent and divalent ions (especially Mg2+) (Yamazaki et al., 1995), reduced glutathione (Gillam et al., 1993), and specifically negatively charged phospholipids (Ingelman-Sundberg et al., 1996). Also, further work on the involvement of cytochrome b5 in the enhancement of testosterone 6β-hydroxylation by P450 3A4 has shown that this may not be due to it being involved in the second
electron transfer step, as was originally believed, since apo-cytochrome $b_5$ can stimulate the rate by a similar amount (Yamazaki et al., 1996; Yamazaki et al., 2001) and references therein. Finally, an additional complication is the fact that the requirements for cytochrome $b_5$ and phospholipids are substrate dependent with P450 3A4 (Shet et al., 1993; Gillam et al., 1995), since both these groups found that these components were necessary for testosterone and nifedipine oxidation, but were not for erythromycin or d-benzphetamine N-demethylation.

1.3.5 P450 3A4 substrate specificity

Both \textit{in vitro} and \textit{in vivo} studies have lead to the discovery that P450 3A4 is capable of metabolising over 60% of the drugs in use today (Wrighton and Stevens, 1992; Guengerich, 1995; Guengerich, 1999), and in a review by Guengerich (Guengerich, 1999) a lists of at least 100 has been compiled. As a result, this P450 is believed to have the broadest catalytic selectivity of any P450 (Guengerich, 1999). For example, P450 3A4 is capable of metabolising both small molecules like acetaminophen (74 Da) and also very large molecules such as cyclosporin A (1201 Da) (Guengerich, 1995). However, this feature cannot be explained purely by a very large active site since many compounds are metabolised both stereo- and regioselectively, \textit{e.g.} the 6β-hydroxylation of testosterone (Lu, 2001) or the N-demethylation of codeine (Guengerich, 1995). Also, several tetra- or pentapeptides have been found to bind quite tightly (low micromolar range) to P450 3A4 as well, especially those with an attached C-terminal amino group. Some of these are enkephalins, but the biological significance has not yet been elucidated (Hosea et al., 2000).

1.3.6 Cooperativity and substrate binding

In addition to the unusual sensitivity to assay conditions and the enormous array of compounds P450 3A4 can metabolise, observations of cooperatively with some, but not all, of its substrates have been reported (Guengerich, 1999). Homotropic cooperativity has been observed for testosterone 6β-hydroxylation, amitriptyline N-demethylation, estradiol 2-hydroxylation, aflatoxin B1 3α–hydroxylation and 8,9-epoxidation, and α-naphthoflavone 5,6-epoxidation (Ueng et al., 1997; Harlow and Halpert, 1998; Hosea et al., 2000). This type of cooperativity is indicated when a plot of rate of reaction against substrate concentration yields a sigmoidal curve, \textit{i.e.}
increasing concentrations of the substrate stimulate activity. In addition, heterotropic cooperativity has been seen with various substrate and effector reaction mixtures. For example, α-naphthoflavone, a synthetic flavonoid, has been shown to stimulate the rate of testosterone 6β-hydroxylation (Harlow and Halpert, 1998), estradiol 2-hydroxylation, aflatoxin B1 8,9-epoxidation (Ueng et al., 1997), and phenanthrene-oxidation (Shou et al., 1994). Also, quinidine was shown to stimulate the rate of warfarin 4' and 10-hydroxylation (Ngui et al., 2001), and diclofenac 5'-hydroxylation (Tang and Stearns, 2001).

The mechanism by which this cooperativity occurs is not well understood. Initially, it was suggested that cooperativity worked through increasing the affinity of P450 for the CPR. This was because of early work with rabbit liver microsomes (contains P450 3A6) which showed that the stimulation of benzo[a]pyrene hydroxylation by α-naphthoflavone resulted in a decrease in the $K_M$ for the CPR but not for the substrate (Huang et al., 1981). In the light of further investigations, however, it seems likely that the CPR plays only a minor role in the cooperativity of P450 3A4, since it was shown that α-naphthoflavone was capable of stimulating 8,9-epoxidation of aflatoxin B1 but also inhibiting the 3α-hydroxylation of this compound (Ueng et al., 1997). If the CPR was the cause of the enhancement a universal increase of activity would be expected, thus this experimental evidence contradicts this theory. Further contradictory evidence is the fact that the 1'-hydroxylation of midazolam was stimulated, but the 4-hydroxylation was either not affected or partially inhibited by α-naphthoflavone and testosterone (Wang et al., 2000).

When the interactions between phenanthrene and α-naphthoflavone were studied something unusual was found. The metabolism of phenanthrene was enhanced by α-naphthoflavone without changing the $K_M$, the 5,6-epoxidation of α-naphthoflavone was partially inhibited without increasing the $K_M$ (Shou et al., 1994). Similar activation/partial or no inhibition kinetics was observed for diazepam with testosterone (Kenworthy et al., 2001), testosterone with either 7-benzyloxy-4-trifluoromethyl-coumarin or 7-benzyloxyquinoline (Lu, 2001), and quinidine with R- and S-warfarin (Ngui et al., 2001). These investigators concluded that substrate and effector (also a substrate in these cases) were simultaneously bound to the active site
of P450 3A4, since both could access the activated oxygen complex. As a result, various kinetic models have been suggested with either two (Shou et al., 1994; Korzekwa et al., 1998; Shou et al., 2001), or three (Hosea et al., 2000; Kenworthy et al., 2001; Lu, 2001) distinct or partially overlapping binding sites within the active site of P450 3A4.

Further evidence in support of multiple substrate binding domains in the active site of P450 3A4 has been derived from site-directed mutagenesis (Harlow and Halpert, 1998). Harlow et al (Harlow and Halpert, 1998) replaced residues Leu-211 and Asp-214 of P450 3A4 with the much larger amino acids Phe and Glu, respectively. The result of this double mutation was that the rate of testosterone metabolism increased, and both homotropic and heterotropic cooperativity, by α-naphthoflavone, was abolished. In addition, the usual cooperative binding observed between testosterone and P450 3A4 was abolished as well. From these data it was concluded that the mutations had blocked the separate effector binding site, thus leading to a loss of cooperativity, but also mimicked the effect of having an effector present, hence the increase in rate of testosterone hydroxylation. However, a later study showed that the 5,6-epoxidation of α-naphthoflavone, an effector and substrate, was only slightly effected by the double mutation (Domanski et al., 2000). This data suggested that α-naphthoflavone was metabolised at a site that was distinct from both the location where it modulates testosterone hydroxylation and from the position where testosterone binds to be hydroxylated. By additionally mutating Phe-304 to a Trp, thus making a triple mutant, it was found that the rate of progesterone hydroxylation was increased, homo- and heterotropic cooperativity had been abolished, and the rate of α-naphthoflavone epoxidation had been reduced by eight fold. Also, it was shown that when just a single mutation was made, Phe-304 to Trp, the epoxidation of α-naphthoflavone was still reduced by over four fold. This work led to the suggestion that residues Leu-211, Asp-214 and Phe-304 were part of an effector binding region which overlapped with the site where α-naphthoflavone binds in order to be metabolised, and that Phe-304 might be act as a contact point for both substrate and effector. Thus, Domanski et al (Domanski et al., 2000) proposed a model for P450 3A4 cooperativity that consisted of two substrate binding sites, which have access to the reactive oxygen intermediate, and an effector binding site, which overlaps with
which overlaps with these binding sites but may not have access to the reactive oxygen intermediate. Recently, the theory that P450 3A4 can bind two molecules simultaneously was given greater credibility, since a pyrene-pyrene complex was observed using fluorescence spectroscopy that could only be within this protein's active site (Dabrowski et al., 2002). Also, it has been found that P450 107, which usually oxidises erythromycin and thus has a large active site, demonstrates homotropic cooperativity towards both androstenedione and 9-aminophenanthrene (Cupp-Vickery et al., 2000). Crystals of this P450 in the presence of these ligands were then grown which indicated that two ligand molecules were present at the active site of P450 107.

However, in addition to multiple binding domains there is some evidence to suggest that P450 3A4 exists in multiple, kinetically distinguishable conformations that have different substrate specificities (Koley et al., 1995). In a later study by Koley et al (Koley et al., 1997), benzo[a]pyrene was shown to bind to an arbitrary conformer I of P450 3A4, but in the presence of α-naphthoflavone it bound preferentially to a conformer II. It was also found that benzo[a]pyrene hydroxylation was stimulated by the presence of α-naphthoflavone. In light of these results Koley et al (Koley et al., 1997) suggested that conformer II was more catalytically active than conformer I, with respect to benzo[a]pyrene hydroxylation, and thus this was the reason that α-naphthoflavone brought about a stimulation of activity. More recently, it has been suggested that individual intermediates in the P450 3A4 catalytic cycle could be considered to be distinct enzyme forms during interactions with substrates (Hosea et al., 2000; Tang and Stearns, 2001).

Generally, a combination of multiple binding domains and alternative conformations seems to provide a plausible mechanism for both homotropic and heterotropic cooperativity. However, these concepts still lack any physical representation and thus further work is required, especially considering that cooperativity has now been demonstrated for other P450s as well, e.g. 105D1 (Taylor et al., 1999), 1A2 (Miller and Guengerich, 2001), and 2C9 (Hutzler et al., 2003). Of these enzymes P450 105D1 could be the most useful in elucidating the mechanism of cooperativity demonstrated by P450 3A4, since it can metabolise similar substrates, is stimulated
and is not membrane bound, thus it could potentially be crystallised. However, it is
worth mentioning at this stage that the clinical significance of cooperativity is still
unclear because so few examples exist in vivo, (Tang and Stearns, 2001) and
references therein, therefore this phenomenon could just be an academic curiosity.
Nevertheless, until all the properties of P450 3A4, and other P450s, are fully
understood the issues associated with drug metabolism and pharmacokinetics are
unlikely to be fully resolved.

1.4 Objectives of this study

1.4.1 Introduction

Obtaining structural information on P450s is crucial for understanding the key
interactions that determine substrate specificity. This knowledge would facilitate
both the development of predictive models for metabolism and toxicity, and the
ability to design drugs with an improved therapeutic effectiveness.

Crystal structures have been obtained for bacterial P450s since these proteins are
highly soluble and can be produced in great quantities. Human P450s, on the other
hand, are membrane associated and are far less soluble and can only be produced in
much smaller amounts. The only mammalian P450s that have been reported so far
are P450 2C5 (Cosme and Johnson, 2000) and P450 2C9 (Williams et al., 2003)
although the company Astex have also crystallised P450 3A4 but its coordinates are
not in the public domain at present (http://www.astex-technology.co.uk). An
alternative method for obtaining structural information would be to assign the protein
residues using NMR spectroscopy. Recently it was reported that virtually all of the
723 residues of malate synthase G (81.4 kDa) had been assigned using various NMR
experiments (Tugarinov et al., 2002). Even though P450 3A4 (60.2 kDa, see
appendix A) is smaller than malate synthase G, it is far less soluble, requires
detergents to stay in solution and has a paramagnetic centre that will broaden the
resonances from the amino acid residues closest the haem, i.e. the active site residues
which are of most interest. All of these factors added together would make it
extremely difficult to obtain any information from similar NMR experiments.
Fortunately however, NMR can be used to determine distances from the haem iron to
substrate protons using an approach called paramagnetic relaxation. Although this technique cannot be used to determine a protein-substrate complex structure directly, the iron-substrate proton distances can be used as constraints for the substrate when docking it into a homology model of the P450. Indeed, this technique has been used successfully in this laboratory to generate haem-iron to substrate proton distances for P450 102 and 2D6 (Modi et al., 1995; Modi et al., 1996a; Modi et al., 1996b; Modi et al., 1997). In addition, other groups have employed this method to study the interaction between P450 1A2 and caffeine (Regal and Nelson, 2000), and P450 2C9 and tienilic acid, diclofenac and lauric acid (Poli-Scaife et al, 1997). Also, preliminary work carried out on P450 3A4 (Modi, 1996, unpublished results) suggested that this technique should work with this protein. Since this method requires only small amounts of protein because the effects are so large, it can potentially offer both sensitivity and precise structural information.

1.4.2 Aims of this project

Originally, the objectives were to:

1) Study substrate binding using both optical and NMR spectroscopy.
2) Use NMR derived haem iron-substrate proton distance data to further develop the in-house model of P450 3A4, which should then be used to design mutants to analyse the important amino acid residues that take part in substrate binding.
3) Determine whether any correlation exists between substrate or product structure and its dissociation constant, \textit{i.e.} its affinity for P450 3A4.

However, during the course of this study it became apparent that emulgen 911 (E911), the detergent used for the solubilisation, inhibited the enzyme, and this was independently corroborated by another group (Hosea and Guengerich, 1998). Thus more basic work was required to be done first, in terms of devising appropriate purification procedures, to obtain fully active protein suitable for spectroscopic studies.
Chapter One

This thesis describes both the work that suggests E911 inhibits substrate binding, and the results and conclusions made about the active samples of P450 3A4 that were studied using NMR and optical spectroscopy.
CHAPTER TWO

Materials and Methods
2.1 Materials

Competent *E.coli* JM109 cells were purchased from Promega. Centrifugal filter units (Centripreps and centricons - Ultracel-YM membrane 30KDa molecular weight cut off) were bought from Millipore. Protean II Mini PAGE apparatus was purchased from Bio-Rad. Wilmad NMR tubes, 5mm royal imperial, were obtained from Sigma.

2.1.1 Chemicals

All solutions were prepared using double-distilled grade water from an Elga Maxima purification unit.

Growth media (bacto-tryptone, bacto-peptone and bacto-yeast extract) were obtained from Oxoid (Unipath Ltd.). The growth supplements ampicillin, thiamine and δ-aminolevulinic acid (δ-ALA) were sourced from Sigma-Aldrich. The buffer constituents potassium phosphate and tris base were obtained from Fisher Scientific while tris HCl, glycerol, DTT and EDTA were purchased from Sigma-Aldrich. Deoxyribonuclease I, ribonuclease-free (DNase I, RNase free) was obtained from Boehringer Mannheim. The NMR buffer constituents deuterium oxide (99.9at.%D) and deuterated DMSO-d₆ (100at.%D) were purchased from GOSS scientific Instruments Ltd. Deuterated glycerol-d₆ (98at.%D) was obtained from Sigma-Aldrich. All detergents were acquired from Sigma-Aldrich apart from emulgen 911 (E911) (Karlan Research Products Corporation). Substrates were obtained from Sigma-Aldrich apart from 7-benzyloxyquinoline (7-BQ) and 7 hydroxyquinoline (7-HQ), which were purchased from BD Gentest.

Chromatography columns (HiTrap chelating sepharose, HiTrap Q and SP sepharose, and HiLoad 26/60 Superdex-200) and Sephadex G25 desalting columns (PD10 and Nap 5) were obtained from Amersham Pharmacia Biotech.
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All other chemicals were from either Fisher Scientific or Sigma-Aldrich and were of at least analytical grade.

2.1.2 Expression Vector

A pCWori vector containing the P450 3A4 gene, an ompA+2 leader sequence and a six histidine tag on the C-terminus was obtained from Dr. Mark Paine, Biomedical Research Centre, University of Dundee, Dundee.

2.2 Methods

2.2.1 Expression and purification of cytochrome P450 3A4

2.2.1.1 Preparation of media, supplements and antibiotics

The growth media were prepared as described below:

LB\textsubscript{amp} media contained tryptone (10 g/L), yeast extract (5 g/L) and sodium chloride (10 g/L). The solids were dissolved in H\textsubscript{2}O and sterilized by autoclaving at 120\degree C for 25 minutes. The media were then cooled to below 60\degree C before the addition of ampicillin, (100 \mu g/mL), prepared as described below. LB\textsubscript{amp} agar also contained agar (15 g/L).

Modified Terrific Broth (TB) media contained tryptone (12 g/L), yeast extract (24 g/L), peptone (2 g/L), glycerol (4 ml/L), and potassium phosphate buffer (KPB) (23.1 g/L \( \text{KH}_2\text{PO}_4 \), 125.4 g/L \( \text{K}_2\text{HPO}_4 \)). For each litre of media, the solids and glycerol were dissolved in 900 mL of H\textsubscript{2}O and sterilized by autoclaving at 120\degree C for 25 minutes. KPB (100 mL) was prepared and autoclaved separately. The liquids were cooled to below 60\degree C and then combined just before use.

Stock solutions of the supplements for the TB growth were made as follows:

The trace elements stock solution (4000x) contained iron (III) citrate (6.1 mg/L), zinc chloride (0.43 mg/L), cobalt chloride hexahydrate (0.5 mg/L), sodium molybdate (0.5 mg/L), calcium chloride (0.25 g/L), copper chloride (0.32 mg/L), boric acid (0.13 mg/L), and concentrated HCl (0.0025% v/v). This solution was filter sterilised.
Isopropylthio-\(\beta\)-D-galactoside (IPTG) (0.2 M), \(\delta\)-ALA (0.5 M), thiamine (1 M) and ampicillin (50 mg/mL) were dissolved in water and filter sterilised.

2.2.1.2 Transformation of *E.coli* JM109 cells with the pCW plasmid

A sample of competent JM109 cells was thawed on ice and separated into 50 \(\mu\)L aliquots. Plasmid DNA (approximately 150 ng) was added to each aliquot and the samples incubated on ice for 30 minutes. A negative control of competent cells with no added DNA was also prepared. The solution was then heat pulsed for 45 seconds at 42°C and afterwards placed on ice for 2 minutes. LB (0.5 mL) was added to the transformed cells and this solution was incubated at 37°C for 1 hour. After this time 100 \(\mu\)L of this solution was added to the centre of an LB\(\text{amp}\) (100 \(\mu\)g/mL) agar plate which was incubated at 37°C overnight.

2.2.1.3 Cell growth, Harvesting and spheroplast formation

From the LB\(\text{amp}\) agar plate, a single colony was used to inoculate a 6 mL solution of LB\(\text{amp}\) and grown at 37°C overnight. From this starter culture 5 mL was taken to inoculate 500 mL of modified TB medium containing ampicillin (50 \(\mu\)g/mL), thiamine (1 mM), trace elements (0.125 mL of stock solution/0.5L) and \(\delta\)-ALA (0.5 mM). The TB culture was grown at 30°C to an optical density at 600nm (OD\(\text{600}\)) of 0.8, induced using IPTG (0.2 mM) and then grown for a further 22-24 hours, final OD\(\text{600}\) of 10-11. The cells were harvested by centrifugation using a Beckman JA-10 rotor at 8000 rpm (11325g) for 20 minutes at 4°C. The supernatant was discarded and the pellet resuspended in Tris.HCl (50 mM) pH 7.6, sucrose (250 mM), and EDTA (0.5 mM). Lysozyme was added to a final concentration of 0.25 mg/mL and the solution was stirred for 30 minutes at 4°C. After this time the solution was centrifuged using a Beckman JA-14 rotor at 8,000 rpm (9820g) for 20 minutes at 4°C, the supernatant was discarded and the spheroplasts were frozen at -20°C.

2.2.1.4 Solubilisation and purification of P450 3A4

The following buffers were used for the purification of P450 3A4:
Buffer A: KPB (20 mM), glycerol (20% v/v), potassium chloride (KCl) (0.5 M), pH 7.6.
Buffer AC: same as buffer A + 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) (10 mM)

Buffer AE: same as buffer A + E911 (0.1% w/v)

Buffer BE: KPB (20 mM), glycerol (15% v/v), KCl (0.5 M), imidazole (1 M), E911 (0.1% w/v) pH 7.6.

Buffer BC: same as buffer BE but with CHAPS (10 mM) instead of E911.

Buffer QE: Tris.HCl (20 mM), glycerol (15% v/v), DTT (1 mM), EDTA (1 mM), E911 (0.1% w/v) pH 7.5.

Buffer QC: same as buffer QE but with CHAPS (2 mM) instead of E911.

Buffer S: KPB (50 mM), KCl (150 mM), glycerol (5% v/v), DTT (1 mM), EDTA (1 mM), CHAPS (2 or 0.5 mM) pH 7.5.

The spheroplasts were thawed at room temperature and resuspended in Buffer A containing phenylmethylsulfonyl fluoride (PMSF) (final concentration of 1 mM) dissolved in ethanol (final concentration 1% v/v), magnesium chloride (5 mM) and DNase I, RNase free (10 units/ml). Typically, the volume used was 1/20th of the original TB culture. This suspension was stirred at 4°C until it was homogeneous (usually 30 minutes) and then sonicated at an amplitude of 10 microns using a Soniprep 150 with the large probe (19 mm diameter) for 15 seconds, after this time it was placed on ice for 90 seconds. This process was repeated 4 times.

At this stage, method A1, 2 or method B was used to purify P450 3A4. Method A1 and A2 used E911 and CHAPS, respectively, as the detergents for the purification but, other than that, they are the same route. Both methods employ the use of a Hi-Trap chelating affinity column as the first step, but method A uses a Hi-Trap Q column as the second step, whereas method B uses a gel filtration column instead.

2.2.1.5 Method A1

E911 (10% w/v stock solution) was added to the sonicated suspension to a final concentration of 0.1% and the solution stirred for 2 hours at 4°C. The suspension was then ultra-centrifuged using a 50.2Ti rotor at 35,000 rpm for 1 hour at 4°C, after which time the supernatant was collected and stored at 4°C until the affinity column was ready for use, normally the following day.
2.2.1.5.1 Hi-Trap chelating affinity column

A Hi-Trap sepharose chelating (5 mL) column was charged with nickel sulphate (10 mL, 0.1M solution) and equilibrated with Buffer AE (25 mL). The supernatant was then loaded onto the column using a peristaltic pump at 4°C, after which the column was connected to a fast protein liquid chromatography (FPLC) system. The elution profile of the column was monitored at 280nm. The column was washed with the following step gradient of imidazole using buffers AE and BE: a) 0 mM (40 mL), b) 30 mM (25 mL), c) 60 mM (25 mL), d) 500 mM (10 mL). The protein eluted in the final step, usually within 3-4 mL, and was then diluted by half with buffer QE and dialysed, using dialysis tubing with a 12 kDa MW cut off, against three changes of a 10 fold larger volume of buffer QE. The conductivity of the protein solution was usually checked at this stage to ensure that the dialysis had gone to completion.

2.2.1.5.2 Anion exchange column

The protein was applied to a Hi Trap Q column (5 mL) that had been equilibrated in buffer QE using an FPLC system at room temperature. The elution profile of the column was monitored at 280nm. The column was washed with the following step gradient of KCl using buffers QE and QE + KCl (1M): a) 0 mM (25 mL), b) 50 mM (15 mL), c) 100 mM (15 mL), d) 200 mM (15 mL), e) 500 mM (15 mL) and finally f) 1 M (15 mL).

2.2.1.6 Method A2

To the sonicated spheroplasts CHAPS (163 mM stock solution) was added to a final concentration of 10 mM, this solution was stirred for 2 hours at 4°C. From this point on the route is identical to method A1 except buffers AC and BC are used on the Hi-Trap chelating affinity column and buffer QC is used for the dialysis and Hi-Trap Q column.

2.2.1.7 Method B

This method is the same as described for A2 except that the protein collected in the final step of the Hi-Trap chelating affinity column was loaded directly onto a
Pharmacia Superdex 200 (26/60) column, which had been equilibrated with buffer S (600 mL), and then eluted in the same buffer.

The Superdex-200 column had been previously calibrated using a Molecular Weight Marker Kit Gel Filtration (MW 29-700kDa) from Sigma. The buffer used was Tris.HCl (0.05 M), potassium chloride (0.2 M), glycerol (5% v/v), pH 7.5. Since buffer S is different to the calibration buffer only approximate molecular weights are reported by using Equation 2-1:

\[
\log MW (\text{Da}) = \frac{574 - V_e}{77}
\]

Equation 2-1

where \( V_e \) = elution volume in mL (peak centre)

### 2.2.1.8 Preparation of stock protein solution for characterisation and storage

The P450 3A4 samples purified by methods A1, 2 and B (where CHAPS (2 mM) was used) were buffer exchanged into KPB (0.1 M) containing glycerol (5% v/v), DTT (0.2 mM) pH 7.5 and either E911 (0.1% w/v) or CHAPS (2 mM) by means of a Centriprep YM30, 30KDa cut off, and a buffer exchange column, Nap 5 or PD 10. The Centriprep YM30 was used to reduce the volume of the sample before addition to the buffer exchange column. The protein prepared by method B (where 0.5 mM CHAPS was used) was exchanged, using just the Centriprep YM30, into KPB (50 mM), KCl (150 mM), glycerol (5% v/v), DTT (1 mM), EDTA (1 mM) and CHAPS (0.5 mM) pH 7.5.

The stock protein solution generated was usually between 50-100 μM, as determined by the method in the following section. The protein was either stored at 4°C until use, which was within one week, or was stored in aliquots of 0.1 mL at -20°C.

By centrifuging the protein solution at 13 krpm for 5 minutes and then performing a P450 concentration determination, see following section, the solubility of P450 3A4 could be assessed over time.
2.2.2 Protein concentration determination

The protein concentration was determined spectrophotometrically using a Perkin-
Elmer UV/Visible Lambda 20 or Cary 300 BIO, with Peltier temperature controller,
at 25°C. The protein was diluted into buffer Z (KPB (0.1 M), glycerol (10 % v/v),
E911 (0.1% w/v) pH 7.5) and reduced with sodium dithionite (5 mg/mL). This
solution was split between two cuvettes and a baseline was taken. The sample
cuvette had carbon monoxide bubbled through it for about 30 seconds after which a
spectrum of the reduced P450 CO bound minus reduced P450, i.e. a difference
spectrum, was taken. By using an extinction coefficient of 0.091 μM^{-1} cm^{-1} (Omura
and Sato, 1964), for the difference between the absorbance at 448nm and 490nm, the
P450 concentration could be calculated.

2.2.3 SDS-polyacrylamide gel electrophoresis

For the analysis of protein solutions, samples containing 0.5-2.5 μg of the protein
solution were added to 10 μL of sample buffer (Tris.HCl pH 6.8 (62.5 mM), glycerol
(10% v/v), sodium dodecyl sulphate (SDS) (2% v/v), 2-mercaptoethanol (5% v/v),
bromophenol blue (0.025% w/v)).

A BioRad Mini-Protein II apparatus was used to perform electrophoresis, employing
a discontinuous gel system consisting of a stacking gel and resolving gel. The
stacking gel contained acrylamide (4.2% w/v) in Tris.HCl pH 6.8 (125 mM), SDS
(0.1% w/v), and the resolving gel contained acrylamide (10% w/v), in Tris.HCl pH
8.8 (375 mM), SDS (0.1% w/v). The gels were run at 200V for 1 hour in an
electrophoresis buffer containing Tris.HCl pH 8.5 (25 mM), glycine (125 mM), SDS
(0.1% w/v).

The gels were stained in an aqueous solution of methanol (45% v/v), acetic acid
(10%v/v), Coomassie Brilliant Blue (0.25% w/v) by microwaving the solution for 30
seconds, and then destained in water until the protein bands were clearly visible.
2.2.4 UV/Visible analysis

A sample of purified P450 3A4 was diluted with KPB pH 7.5 (100 mM) to a concentration of 1 μM and scanned between 300-500 nm using a dual beam Cary spectrophotometer at 25°C against a reference cuvette containing only diluent.

2.2.5 N-Terminal sequencing

This work was carried out by Protein and Nucleic Acid Chemistry Laboratory (PNACl) at Leicester University (http://www.le.ac.uk/cmht/pnacl/pnacl.html) using an ABI 476 protein sequencer. A sample of protein (3 μg) was electrophoresed through an SDS-polyacrylamide gel, rinsed with water and given immediately to PNACL for analysis.

2.2.6 Activity assay

Fluorescence experiments were carried out on a Perkin-Elmer LS 50B Luminescence Spectrometer. A solution of 7-benzyloxyquinoline (7-BQ) (0.5 mM) in methanol (2% v/v) was prepared in KPB (100 mM), glycerol (5% v/v) pH 8.0 to which was added 1 μM of P450 3A4. This solution was equilibriated at 25°C for 3 minutes after which the reaction was initiated with cumene hydroperoxide (1 mM), which was dissolved in methanol at a concentration of 100 mM. The reaction mixture contained a final methanol concentration of 3% v/v. The fluorescence at 530 nm (slit width 20 nm) was observed when exciting at 409 nm (slit width 2.5 nm) using a 1 cm path length cuvette. Initial rates were obtained from the first 10 seconds of data after reaction initiation and converted into rates of 7-HQ formation/nmol/min/nmol P450.

2.2.7 Determining the percentage high spin of P450 3A4 samples in the absence and presence of a substrate using mathematical simulations derived from P450 101

The raw spectral data for P450 101 in a 0% and 100% high spin state were obtained from Dr A. Westlake.

By using the following equation it was possible to obtain spectral simulations of different percentage high spin states.
\[
\%HS = \left[\left(\%HS \times SD_{hs}\right) + \left((100 - \%HS) \times SD_{ls}\right)\right]/100
\]

Equation 2-2

where \%HS, SD_{HS} and SD_{LS} are percentage high spin, 100% high spin spectral data and 0% high spin spectral data, respectively.

In order to determine the percentage high spin content of a sample of P450 3A4 it was first necessary to obtain the 390/418 value, which was determined by dividing the optical density at 390nm by that at 418nm. By then producing a mathematical simulation of P450 101 with the same 390/418 value, normally to within +/- 0.01, it was possible to estimate the percentage high spin content of that sample of P450 3A4.

2.2.8 Determination of substrate binding constants, \(K_d\) using absorption spectroscopy

2.2.8.1 Experimental technique

The interactions of various compounds with P450 3A4 were determined employing absolute spectroscopy using a dual beam Cary Spectrophotometer. The protein was diluted into various different buffer solutions, see Chapter four and five, to a final concentration of 1 or 2\(\mu\)M within a 1cm path length cuvette, the initial sample volume was always 900 \(\mu\)L. Absolute spectra were recorded from 450-350nm against a reference cuvette that contained the same buffer only, at 25°C, unless otherwise stated. Substrates that were found to absorb in the region of interest were also titrated into the reference cuvette, using the same method as for the sample cuvette, in order to remove this absorbance from the spectrum. Three titration methods were employed during this investigation:

1) Aqueous soluble substrates were dissolved in buffer and titrated into the sample cuvette, diluting it to a maximum of 35%.
2) Substrates that were not aqueous-soluble were dissolved in an organic solvent, normally dimethylsulphoxide (DMSO), and then diluted into buffer to give a
final DMSO concentration of 2% v/v. A solution of protein was prepared containing the organic solvent, at the same concentration, in the sample cuvette and then the substrate was titrated into it in the same way as for aqueous substrates.

3) For substrates with very low solubility, an alternative method was employed. A solution containing both protein and DMSO (2 or 5% v/v) was added to the sample cuvette. In addition, a stock solution of substrate and protein was prepared in buffer with DMSO (2 or 5% v/v). By removing an aliquot from the sample cuvette, and replacing it with an equal volume of the stock solution the substrate concentration could be increased while maintaining the sample volume and protein concentration.

2.2.8.2 Data analysis

Data analysis was performed upon the difference spectra, which were obtained by taking each optical spectrum and subtracting the first spectrum from it. The change in absorbance (ΔA) was determined by subtracting the absorbance minima (trough) from the absorbance maxima (peak). The binding constant, $K_d$, and maximum absorbance change for the transition, $ΔA_{max}$, were determined using the standard quadratic Equation 2-3 that assumes a single binding site. A more general form of this equation, Equation 2-4, was also used to obtain the substrate concentration at half-maximal binding $K_{0.5}$ and an indication as to whether there may be cooperativity. An $n$ value of one suggests a single binding site. If the $n$ value is above one this indicates cooperativity. The analysis and fitting were done using the program Origin, version no. 6.

\[
ΔA = \frac{ΔA_{max}}{2 \times E} \left[ E + S + K_d - \left( (E + S + K_d)^2 - 4 \times E \times S \right)^{\frac{1}{2}} \right]
\]

Equation 2-3

\[
ΔA = \frac{ΔA_{max} \times S^n}{K_{0.5}^n + S^n}
\]

Equation 2-4

where $E$ and $S$ are P450 3A4 and substrate concentration, respectively.
Chapter Two

2.2.9 NMR Studies

2.2.9.1 General

All proton nuclear magnetic resonance ($^1$H NMR) experiments were carried out on a 500MHz Bruker DMX spectrometer at 300K, unless otherwise stated. Proton chemical shifts were referenced to HDO at 4.75 ppm.

$^1$H resonance assignment of substrates was carried out by performing both 1D and 2D COSY experiments. In the case of testosterone it was also necessary to carry out some homonuclear decoupling experiments, these were performed on a 400MHz Bruker DMX spectrometer at 300K.

Buffers were prepared at twice their desired concentration and pH corrected in $^1$H$_2$O, they were then freeze-dried. When required the buffer was re-hydrated in $^2$H$_2$O and the pH* checked (pH* means a pH meter reading uncorrected for isotope effects on the glass electrode).

Substrates were dissolved in $^2$H$_2$O or deuterated DMSO d$_6$ and diluted to their final concentration in the chosen buffer.

The protein was buffer exchanged into KPB (100 mM), glycerol d$_8$ (5% v/v) pH 7.5 and either E911 (0.1% w/v) or CHAPS (2 mM or 0 mM) in $^2$H$_2$O using either a NAP-5 or PD10 column, unless stated otherwise.
2.2.9.2 Determining $K_d$ for interaction between testosterone and 2β-
hydroxypropyl cyclodextrin

2.2.9.2.1 Experimental technique

An NMR sample was prepared by diluting a stock solution of testosterone (8 mM), in
DMSO $d_6$, 20 fold into KPB (0.1 M KPB), glycerol $d_8$ (5% v/v) pH 8.0, so as to yield
a final testosterone and DMSO concentration of 0.4 mM and 5% v/v, respectively.

Two stock solutions of 2-hydroxypropyl β-cyclodextrin (2.5 and 12.4 mM) were
then prepared in KPB (0.1 M KPB), glycerol $d_8$ (5% v/v), DMSO $d_6$ (5% v/v), pH
8.0, which contained testosterone (0.4 mM).

A 1D $^1$H spectrum was obtained of the NMR sample. Following this the NMR
sample was titrated with the solution containing the lower stock concentration of
cyclodextrin and after each addition a 1D $^1$H spectrum was obtained. Once the
cyclodextrin concentration reached 0.57 mM the solution containing the higher stock
concentration of cyclodextrin was used to complete the titration.

2.2.9.2.2 Data Analysis

Since the batch of 2-hydroxypropyl β-cyclodextrin had a mean degree of substitution
of 5, i.e. on average each molecule of cyclodextrin has 5 hydroxypropyl groups on it,
its molecular weight was determined to be 1430 Da.

The fitting of the data was performed using Equation 2-3 within the program Origin,
version no. 6.

2.2.9.3 Paramagnetic relaxation

2.2.9.3.1 Experimental technique

The spin-lattice relaxation time ($T_1$), or rate ($R_1 = 1/T_1$), was measured by an
inversion recovery ($180^\circ-\tau-90^\circ$) experiment (Sass and Ziessow, 1977), modified to
include pre-saturation of the water signal in order to improve the signal-to-noise ratio (see Appendix B).

The 90° pulse width was calibrated first using a 1D experiment. A series of 1D experiments were performed, usually at least 12, with an ever increasing time delay, \( \tau \) value, (0.00005-15s) and these experiments were collected in the 2D mode. Once the 90° pulse was known for the system under investigation, the following set of experiments were carried out:

1) The substrate protons’ relaxation rates in the absence of protein, \( R_{1,f} \) s, were ascertained using the method described in section b) data analysis.

2) Individual samples containing the protein at a fixed concentration were prepared with increasing amounts of substrate. As in 1) the \( R_{1,obs} \) values for the substrate protons were determined.

The stability of the protein was checked at the end of particularly long experiments (2 hours or more) by determining the P450 concentration.

**2.2.9.3.2 Data analysis**

Dipolar interactions between the substrate protons and the unpaired electrons on iron (III), a paramagnetic centre, at the active site of P450 3A4 bring about an increase of the spin-lattice relaxation rate (\( R_1 \)) of the substrate protons. \( R_1 \) can then be used in Equation 2-8 to calculate the distance of a particular proton from the iron.

The \( R_{1,f} \) or \( R_{1,obs} \) values were obtained by fitting Equation 2-5 to the measured peak height as a function of the delay, \( \tau \):

\[
I(t) = I(0) + P \cdot \exp(\tau R_1)
\]

Equation 2-5

Assuming that the bound substrate is exchanging rapidly with those in the bulk solution, a fast-exchange regime, then the observed spin-lattice relaxation rate represents the weighted average between these two states (Modi et al., 1996), *i.e.* bound and free (\( R_{1,b} \) and \( R_{1,f} \), respectively):
where \( p_f = [S_f]/[S_0] \) and \( p_b = [S_b]/[S_0] \) are the fraction of the substrate in the free and bound state, respectively, and \([S_f], [S_b] \) and \([S_0] \) are the free, bound and total substrate concentrations, respectively. When using a great excess of substrate over enzyme, as in the present case, \( p_f \approx 1 \) and the following equation can be derived:

\[
R_{1,obs} = p_f R_{1,f} + p_b R_{1,b}
\]

**Equation 2-6**

\[
R_{1,obs} = \frac{[E_0]}{K_d + [S_0]} \left( R_{1,b} - R_{1,f} \right) + R_{1,f}
\]

**Equation 2-7**

where \([E_0] \) is the total enzyme concentration and \( K_d \) is the binding constant.

Equation 2-7 can be used in two ways to determine the \( R_{1,b} \) value for a given proton:

1) \( R_{1,obs} \) values are plotted against substrate concentration, \([S]\), for a particular proton resonance and \( R_{1,b} \) and \( K_d \) can be calculated using non-linear regression.

2) \( R_{1,obs} \) values are plotted against \( E_0/(K_d + S_0) \) and the slope of the line yields \( R_{1,b} \), although in this plot to be used the \( K_d \) must be known, since this is not always the case the first method is used more often.

\( R_{1,b} \) is related to the paramagnetic relaxation rate \( (R_{1,M}) \) and the off rate \( (k_{off}) \) of the system. When the system is in fast exchange, however, \( k_{off} \) is much greater than \( R_{1,M} \) and so \( R_{1,b} \) approximates to \( R_{1,M} \). The fast exchange condition can be verified by observing the temperature dependence of \( R_{1,b} \), since \( R_{1,M} \) and \( k_{off} \) have opposite temperature dependencies. \( R_{1,M} \) decreases with increasing temperature, as the relaxation mechanism becomes less efficient, and \( k_{off} \) increases with increasing temperature since the complex dissociates at a faster rate. A less accurate method of determining the exchange regime is to observe the temperature dependence of \( R_{1,obs} \) values, which should result in the same conclusions as above assuming the \( K_d \) of the system does not change significantly with temperature.
The relationship between $R_{i,M}$ and the distance of the proton is described by the Solomon-Bloembergen equation (Solomon and Bloembergen, 1956; Bloembergen, 1957):

$$R_{i,M} = \frac{2}{15} \left( \frac{\mu_0}{4\pi} \right)^2 \gamma_I^2 g^2 S(S+1) \beta^2 \left( \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2} \right) + \frac{2}{3} S(S+1) \left( \frac{A}{\hbar} \right)^2 \left( \frac{\tau_c}{1+\omega_S^2\tau_c^2} \right)$$

Equation 2-8

where $\mu_0$ is the vacuum permeability constant, $\gamma_I$ is the nuclear giromagnetic ratio, $g$ is a constant that describes the electronic environment, $S$ is the total electron spin, $\beta$ is the Bohr magneton, $\omega_I$ and $\omega_S$ are the nuclear and electronic Larmor frequencies, respectively, $\tau_c$ and $\tau_e$ are correlation times that describe the fluctuations of the dipolar and scalar interactions, respectively, $r$ is the distance between the nucleus and the electron, and $A$ is the scalar interaction constant. The first term is the dipolar and the second term the scalar contribution to the spin-lattice relaxation time. The scalar term can usually be neglected, since the $A/\hbar$ term, where the value is known, is often in the order of $10^6$ Hz, whereas the coefficient of the dipolar term, which contains $\beta^2$, is of the order of $10^{12}$ Hz, thus its value is much smaller (Jardetzky and Roberts, 1981).

The correlation time $\tau_c$ can be determined by using equation 2-8:

$$\tau_c^{-1} = \tau_r^{-1} + \tau_s^{-1} + \tau_m^{-1}$$

Equation 2-9

where $\tau_r$ is the rotational correlation time, $\tau_s$ is the electron spin relaxation time, and $\tau_m$ is the time for chemical exchange (Bertini et al., 1989). The value of $\tau_c$ will approximate to the fastest process in the solution, and in the case of heamoproteins this is usually the electron spin relaxation time, which is usually in the order of $10^{11}$-$10^{10}$ s (Bertini et al., 1989).

2.2.9.3.3 Determining effective S number and theoretical $R_{i,obs}$ values

In order to predict $R_{i,obs}$ values it is necessary to determine an effective S number, since P450 3A4 does not form 100% high spin complexes with its substrates. By
using the method described in section 2.2.7 the percentage high spin present in a P450 3A4-substrate complex can be estimated, and this value can then be applied to the following equation to obtain the effective S number for this system:

\[
S_{\text{eff}} = \frac{\left(\frac{\%HS \times 5}{2}\right) + \left(100 - \%HS\right) \times \frac{1}{2}}{100}
\]

**Equation 2-10**

where \(S_{\text{eff}}\) and \(\%HS\) are effective S number and percentage high spin, respectively.

Once the effective S number is known the \(R_{1,M}\) can be determined since all the other values in equation 2-8 are known constants except for the correlation time \(t_c\), which was estimated to be \(2 \times 10^{-10}\) s since this is the value obtained for P450 2C9 (Poli-Scaife et al, 1997).

Thus equation 2-8 simplifies to the following:

\[
\frac{R_{1,M} \times r^6}{S_{\text{eff}} (S_{\text{eff}} + 1)} = \frac{2}{15} \left(\frac{\mu_0}{4\pi} \times \gamma_i \epsilon \beta\right)^2 \times \frac{3\tau_c}{1 + \omega_i^2 \tau_c^2} + \frac{7\tau_c}{1 + \omega_5^2 \tau_c^2} + \frac{2}{3} S(S + 1) \left(\frac{A}{h}\right)^2 \frac{\tau_c}{1 + \omega_5^2 \tau_c^2}
\]

\[
= 3.3 \times 10^{-44} + 4.3 \times 10^{-10} \approx 0
\]

therefore

\[
R_{1,M} = \frac{1.4E - 53 \times S_{\text{eff}} (S_{\text{eff}} + 1)}{r^6}
\]

**Equation 2-11**

By using equation 2-11 it is possible to determine theoretical \(R_{1,M}\) values for a range of substrate proton-iron distances at a particular high spin state of P450 3A4. Thus theoretical \(R_{1,\text{obs}}\) values can be obtained by using equation 2-7, since the \(K_d\) and \(R_{1,f}\) values are known and \(E_0\) and \(S_0\) can be estimated.
CHAPTER THREE

P450 3A4 expression, purification and characterisation
Chapter Three

P450 3A4 expression, purification and characterisation

3.1 Introduction

The main aim of this project was to obtain distances between the protons on a substrate and the haem iron using NMR so that a model could be produced to investigate substrate binding to P450 3A4. During the course of the project it was found that the preliminary NMR experiments that were carried out in this laboratory several years ago (Modi, 1996, unpublished results) could not be reproduced. As a result, an alternative detergent and route of purification were investigated for the preparation of P450 3A4.

This chapter will compare and discuss:

1) the two main routes of purification used
2) the characterisation of the samples of P450 3A4 obtained.

Chapter Four will then report on the data obtained from optical binding and NMR experiments carried out on the various samples of P450 3A4.

3.1.1 Structural features of microsomal P450s that interact with the membrane

Microsomal P450s, e.g. P450 3A4 and 2D6, are highly hydrophobic proteins and associate with intracellular membranes (Gonzalez, 1990). Therefore, it would seem sensible to perform the NMR experiments upon a sample of P450 3A4 that is either still associated to the membrane or one that has been purified, but then reconstituted with phospholipids. However, to do this would make it difficult to study the effects of the protein on substrate protons' spin-lattice relaxation rates, $R_1$s. This is because of three reasons:

1) The membranes/phospholipids will give rise to broad resonances in the spectrum, and these could overlap with the substrate proton resonances, which would make it extremely difficult to get an accurate value for that proton's $R_1$. 
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2) The substrate-P450-membrane complex formed would be very large and as a result would give rise to significant line broadening effects, which could cause substrate proton resonances to overlap with one another, and thus make it very difficult to get accurate values for $R_1$ for a particular proton resonance. In addition, the substrate proton's $R_1$ values would be affected purely because of being part of such a large complex (see Chapter Four, section 4.1.3.1).

3) The substrate could interact with the membranes, since many P450 substrates are hydrophobic in nature, which would affect the substrate protons' $R_1$s as a function of the membrane concentration.

Resonances brought about because of the membranes that are associated to P450 3A4 could not be removed, but phospholipid resonances could be. This could be achieved by using deuterated phospholipids, which do not produce $^1$H NMR resonances, but these are very expensive and are often difficult to work with because they are labile. The effects on $R_1$ described in 2) and 3) could, however, be accounted for by carrying out additional experiments where the protein is in the diamagnetic state, in this case the changes observed in $R_1$ would be solely due to non-paramagnetic effects, although these corrections could lead to a less accurate distance. As a result, the NMR experiments that have been performed in this lab with P450 3A4 (Modi, 1996, unpublished results) and P450 2D6 (Modi et al., 1996; Modi et al., 1997) have been carried out with detergent solubilised and purified protein.

Although microsomal P450s have frequently been shown to be catalytically active after having been solubilised with detergents, and then reconstituted with phospholipids and cytochrome P450 reductase (Modi et al., 1996; Miller and Guengerich, 2001; Poli-Scaife et al, 1997), the effect of disrupting these protein-membrane interactions on the protein is not clearly understood. Since reconstituting optimal conditions for the catalytic activity of the P450 3A family is particularly difficult, (Guengerich et al, 1986; Schwab et al., 1988; Elshourbagy and Guzelian, 1980) it is important to understand which parts of the protein interact with the membrane, as they could be affected by detergent solubilisation.

Eukaryotic P450s are all encoded in the nucleus and synthesised in the cytoplasm, but they are incorporated in either mitochondria or the endoplasmic reticulum. Therefore,
the signals that direct the polypeptide to the right compartment within the cell, as well as the signals that govern protein folding, are most likely determined by the amino acid sequence of each polypeptide.

By using various techniques including site-specific antibodies (De-Lemos-Chiarandini et al., 1987), sequence homology modeling with P450 101 (Poulos et al., 1986) and P450 102 (von Wachenfeldt and Johnson, 1995) and genetic engineering (Monier et al., 1988; Szczesna-Skorupa et al., 1988; Ahn et al., 1993; Murakami et al., 1994) it has been shown that the N-terminal region of microsomal P450s contains a signal peptide sequence for membrane insertion. However, attempts to make microsomal P450s soluble by removing this signal peptide have only met with limited success (Pernecky et al., 1995; von Wachenfeldt and Johnson, 1995). When the NH₂-hydrophobic sequence of P450 2A4 was replaced with an amphipathic peptide a peripheral membrane protein was produced, i.e. it could be solubilised using just sodium carbonate instead of using detergents, but it was still highly aggregated in solution (Sueyoshi et al., 1995). In addition, when rat P450 1A1 was expressed in yeast without the hydrophobic N-terminal sequence, it was still found attached to the microsomes and to have the same restricted mobility as the full-length form (Ohta et al., 1994). These data led to the conclusion that for most microsomal P450s the signal peptide is not the only region of the molecule that anchors the protein to the membrane, and that the extent to which these other anchors interact with the membrane is different from one P450 to another. Upon the construction of a detailed molecular model for P450 19, which was based upon the three-dimensional structure of the haem domain of P450 102, it could be seen that the most hydrophobic region at the surface of the molecule was the entrance to the substrate access channel (Graham-Lorence and Peterson, 1995). From this model it was concluded that the regions that could be partially buried in the membrane included parts of the β1 and β2 sheets, the β' helix, and the F-G loop. The fact that no antibodies have been shown to bind in these regions of the membrane-bound P450s, except the β' helix, gives some credence to this model (von Wachenfeldt and Johnson, 1995).

To date, only one microsomal P450's crystal structure has been published (Williams et al., 2000a). This was achieved by deleting the N-terminal membrane-spanning
domain of P450 2C5 and then by making five substitutions in the FG region derived from P450 2C3. It is believed that the effect of making these substitutions in the FG region was to prevent self-aggregation of the molecule once it was soluble (Cosme and Johnson, 2000; Williams et al., 2000b). In addition, mutagenesis work on P450 7A1 has shown that reducing the hydrophobicity of the F-G loop region gives rise to mutants that are more soluble, demonstrated by the fact that a higher content is found in the cytosol rather than the membrane (Nakayama et al., 2001). Thus, it appears likely that the FG region of P450 3A4, a part of the P450 believed to be involved in substrate access to the enzyme, will be involved with membrane binding and self-aggregation of the P450 once it is solubilised. Therefore, if P450s self-aggregate around the postulated substrate access channel, once they have been removed from the membrane, this could affect the kinetics of substrate binding, which is certainly pertinent to the present investigation.

3.1.2 Solubilisation and purification of membrane bound P450 proteins

This section provides a brief summary of how microsomal P450s, e.g. P450 3A4, have been purified in the past, with particular emphasis on the use of E911 or CHAPS, and the problems that have been overcome. It will also explain why non-ionic detergents, which are known inhibitors, became a part of the purification protocol for P450 3A4.

Initially microsomal P450s were only studied within their native environment i.e. microsomal suspensions. However, this meant that instead of investigating how an individual P450 functioned, researchers were in fact studying mixtures of many P450s and the rest of the monoxygenase system, which is mainly cytochrome P450 reductase and the phospholipid membrane (Lu and Coon, 1968). Thus, their results could have been complicated by interactions between the P450 and either the CPR or the phospholipids (Ingelman-Sundberg et al., 1996). In addition, it has been reported that some P450s can interact with others (Yamazaki et al, 1997) and that cytochrome bs, a protein that is also present in liver microsomes (Garfinkel, 1957), can affect the catalytic activity of some P450s, especially P450 3A4 (Hayashi et al., 2000; Yamazaki et al., 1996b; Yamazaki et al., 1996a; Yamazaki et al., 2001).
Thus, the next step was to break down the monooxygenase system into its individual components, so that P450s could be studied in a more defined environment. In order to achieve this, P450s had to be solubilised from the membrane to which they were bound. Early attempts using detergents, low pH, proteases and organic solvents resulted in the formation of an inactive form of P450 called P420, (Omura and Sato, 1964b; Sato et al, 1973). This inactive form of P450 can be observed in the difference optical spectrum when a protein sample is reduced and carbon monoxide (CO) added, since it absorbs at 420nm, hence P420. In 1967, however, the stabilizing effects on detergent solubilised P450 of glycerol and other polyols were discovered (Ichikawa and Yamano, 1967). As a result, solubilising membrane associated P450s with detergents in the presence of glycerol became a standard approach.

3.1.2.1 Introduction to detergents

Detergents are a class of molecules that have an amphiphilic structure, *i.e.* they contain both hydrophilic and hydrophobic moieties. Due to their dual nature, they align themselves at the interface between air (or oil) and water, and this brings about a decrease in the surface tension of the solution as compared with the pure solvent. Detergents are sometimes called surfactants because of this property, since this name is derived from the phrase "surface active agent". This surface activity leads to the solubilisation of hydrophobic molecules into the aqueous medium, a process that is assisted by the formation of detergent micelles around the hydrophobic entities.

Micellisation is the self-aggregation of detergent monomers. At low concentrations, detergents exist as monomers, but above a characteristic limit, called the critical micelle concentration (CMC), micelles are formed. The reason for this micellisation is that at, or above, the CMC these hydrophobic monomers are unable to break the water-water hydrogen bonding interactions that exist in the solution, and thus are forced to self-aggregate; this is called the hydrophobic effect (Southall et al., 2002). Another important factor to affect micellisation is the critical micelle temperature (CMT) (Neugebauer, 1990).

The phase diagram in Figure 3-1 demonstrates that a detergent can only exist as either
crystals, i.e. it is insoluble, or as a monomeric species below the CMC and CMT. It also indicates that micellisation occurs over a narrow concentration range. Although this is true for many detergents, it appears to be inaccurate for those containing bulky, rigid hydrophobic portions, such as the bile acids (Kratohvil, 1983). These detergents can form micelles over a very wide range of concentrations and variations in monomer concentration can occur above the CMC.

3.1.2.2 Principle of detergent solubilisation of membrane bound proteins

It is believed that detergent solubilisation of integral membrane proteins takes place in four stages (Neugebauer, 1992):

1) Detergent binds to the membrane
2) Membrane lysis occurs when the detergent concentration increases beyond a particular threshold.

3) Detergent-lipid-protein complexes form over time, as the membrane becomes solubilised.

4) As the detergent concentration increases further, these complexes are disrupted to produce detergent-protein and detergent-lipid complexes.

It is also believed that detergents must be in a micelle before solubilisation of membrane proteins can occur. Thus, the most important factors when choosing a detergent are its CMC and CMT. Other factors that can affect the detergent's state are pH, ionic strength and additives such as multivalent ions and organic solvents.

The effect of pH on E911 or CHAPS is minimal, since E911 is a non-ionic species and CHAPS, which is a zwitterionic detergent, has no titratable groups within the range of pH used in this study. Ionic strength affects the CMC's of both these detergents. It has been found that as the ionic strength increases, the CMC decreases for both CHAPS (Neugebauer, 1992) and E911 (Dawson et al., 1986). In both cases, the increase in ionic strength results in the water preferentially solvating the ions rather than the detergent, and so a lower concentration of either E911 or CHAPS is required before self-aggregation occurs. In the case of CHAPS there is an additional effect, which is that the local electrostatic repulsion between CHAPS molecules becomes masked, and the hydrophobic effect dominates (Tanford, 1980; Schick, 1967).

Multivalent ions are unlikely to cause problems with E911 or CHAPS, since they normally only precipitate detergents that contain a carboxylate group (Small, 1971). Organic solvent additives could cause the detergents to precipitate or prevent micellisation from occurring and thus should be minimised to a large degree (Neugebauer, 1992).
3.1.2.3 Solubilisation of P450s

Although many detergents are commercially available (Neugebauer, 1992), not all of them are suitable for the purification of P450s. The choice of detergent involves the consideration of two important factors:

1) Its ability to solubilise P450
2) How inhibitory it is to the P450, since many detergents inhibit and are difficult to remove.

Initial studies involving liver microsomes required the preferential solubilisation of P450 over its CPR partner, cytochrome b₅ and epoxide hydrase (Lu and Levin, 1972). It was found that non-ionic detergents, e.g. Triton X-100 and E911, were superior to sodium cholate, an ionic bile salt from which CHAPS was synthesised (Hjelmeland et al, 1983), in this respect (Sato et al, 1973; Lu and Levin, 1972). Due to the improved purity of P450 obtained by using non-ionic detergents, they became an integral part of the purification procedure. However, Denk et al (Denk et al., 1971) found that at low concentrations of non-ionic detergent e.g. Triton X-100 the rate of metabolism of a particular substrate, i.e. aminopyrine, was competitively inhibited. At slightly higher concentrations of the detergent, the P450's ability to bind substrates was reduced, but no P420 could be detected. At yet higher concentrations, the P450 was converted to P420. In another study, where the effect of a range of detergents was measured on the catalytic activity of rat liver microsomes, it was found that Triton N-101, which is structurally similar to E911 (Hosea and Guengerich, 1998), produced marked inhibition in comparison to sodium cholate (Lu and Levin, 1974).

Although non-ionic detergents were inhibitory they were able to generate purer samples of P450, and so methods for removing these detergents were investigated. Unfortunately, it was soon discovered that they could not be removed very easily. The concentration of E911 cannot be reduced by prolonged dialysis because it forms very large micelles (Dawson et al., 1986), which could be as large as the P450. It was also found that a size-exclusion column could remove no more than 50-60% of the E911 from a P450 sample, even when passed through several times (Gaylor and Delwiche, 1969). A more effective method was to bind the P450 of interest onto an
hydroxyapatite column and then remove the non-ionic detergent by repeatedly washing it with a buffer containing sodium cholate instead (Guengerich et al, 1986), since sodium cholate forms far smaller micelles (Hjelmeland et al, 1983) and can be removed by prolonged dialysis (Autor et al., 1973).

3.1.2.4 Introduction to protein purification

Numerous methods exist to purify proteins and this section will cover the approaches employed within this study.

3.1.2.4.1 Principle of nickel chelating affinity chromatography

Nickel chelates basic nitrogen compounds such as imidazole, i.e. the functional group of the histidine amino acid, extremely well and so, when bound to a resin will retard the movement of a tagged protein. By using a competing agent, e.g. imidazole or histidine, bound proteins can be eluted from the resin. By using a concentration gradient of the competing agent, the tagged protein can be separated from other proteins that have become bound to the column, as they are unlikely to have the same high density of histidine residues on the surface and so will bind less tightly. This often leads to a very pure protein sample in a single step (Sriwanthana et al., 1994; Kroiher et al., 1995; Xu et al., 2001).

3.1.2.4.2 Principle of ion-exchange chromatography

This technique separates proteins based upon their surface charge. The pH at which a protein has zero net surface charge is called the pI value. Above this pH the protein is negatively charged and below it is positively charged. If the protein is stable at a pH where it is negatively charged, then an anion exchange column would be used e.g. an HiTrap Q column which has a quaternary ammonium head group. If the protein is stable at a pH where it is positively charged, then a cation exchange column would be used e.g. a HiTrap SP column that has a sulphonate head group. HiTrap Q and SP columns are strong anion and cation exchangers, respectively, which means that they have a fixed positive or negative charge that does not change with pH. Diethylaminoethyl (DEAE) and carboxymethyl (CM) columns are weak anion and cation exchangers, respectively, which means that their positive or negative charges vary with the pH of the buffering solution.
The pI can be determined experimentally using a technique such as chromatofocusing (Sluyterman and Elgersma, 1978; Giri, 1990) or predicted theoretically, by inputting the primary amino acid sequence into a computer program (http://ca.expasy.org/tools/pi_tool.html). The value obtained from the theoretical prediction is based on the assumptions that all the ionisable groups on the protein are exposed and that the local environment does not affect their pKas. These assumptions may be true for a small peptide, where there is not a significant amount of secondary structure, but for a large protein such as P450 3A4 this is unlikely to be the case, and therefore the value obtained can only be taken as a rough guide.

Experimentally, P450 3A4 has been found to flow through a HiTrap Q column at pH 7.5 (Pritchard et al., 1997) and bind to a weak cation exchange column at pH 6.5 (Gillam et al., 1993), which would suggest that the pI is 7.5 or higher. Theoretically, the pI value of P450 3A4 has been calculated to be 8.3 using the above web site program, see Appendix A.

3.1.2.4.3 Principle of size-exclusion chromatography

Size-exclusion chromatography separates proteins based on their hydrodynamic diameter. It is unlike other chromatographic methods because it is not designed to retain proteins i.e. there are usually no binding interactions. Although with some column materials, e.g. Sephadex, there can be weak ionic interactions with proteins, and thus salt is added to prevent this from happening. Since there are usually no binding interactions, limited resolution is obtainable, but fragile proteins will not be damaged by possibly deleterious interactions.

Porous beads are used within the column; these permit molecules of only a certain range of sizes to enter and exclude those that are greater than this. As a result, protein molecules that are larger than the pore size will be eluted first, since they flow straight through the column. Smaller proteins, however, which can enter the pores to varying extents, will be separated into similarly sized groups and emerge later. The smallest molecules will be eluted last.
3.1.2.5 Purification of P450 3A4

When P450 3A4 was first purified, from human liver microsomes in 1986 (Guengerich et al, 1986), sodium cholate and E913 were used in the solubilisation procedure, as described above. Gillam et al (Gillam et al., 1993) purified P450 3A4 from E.coli cells by a similar method and were able to produce 70% pure protein by using a weak anion exchange resin, diethylaminoethyl (DEAE), and a hydroxyapatite column. Domanski et al (Domanski et al., 1998) were the first to avoid using E911 or E913 entirely, by employing CHAPS as the solubilising agent instead. In addition, by using histidine tag methodology, they were able to obtain very pure active protein in just a single step by using a metal chelating affinity column. The CHAPS was removed while the protein was bound to the affinity column, by washing with large volumes of buffer without detergent.

Although all these procedures involve a step that is designed to remove as much detergent as possible, when P450 3A4 is actually used for kinetic assays it is normally reconstituted back into a system containing phospholipids and a small amount of detergent, either cholate or CHAPS (Ueng et al., 1995; Ueng et al., 1997; Harlow and Halpert, 1998; Domanski et al., 1998; Yamazaki et al., 1995). At this stage, the detergents are used to assist protein incorporation into the lipids (Ingelman-Sundberg and Glaumann, 1980).

In order to carry out the intended NMR experiments it was essential to have a sample free of other P450s or haem containing proteins. Purifying native P450 3A4 from human liver microsomes would have resulted in other P450s being present. E.coli, however, produces no other P450s and so this host was used to express recombinant P450 3A4. An additional benefit to using a recombinant protein is that it is possible to add a histidine tag, thus making the purification easier.

It should be noted that the present study requires the preparation of a stock protein solution that is still active when it is diluted into a buffer containing only glycerol as a stabilising agent. If phospholipids or detergents were used within the NMR sample at the concentrations employed by other research groups, then the substrate’s resonances
would be overlapped and broadened and reliable data would be very difficult to obtain.

3.1.3 Optical characterisation and catalytic activity of P450 3A4

The following sections will provide a background to both the optical characterisation and the catalytic activity experiments that have been carried out on the samples of P450 3A4 that were produced.

3.1.3.1 Optical characterisation of P450s

P450s gain their characteristic optical signature from the fact that they possess an iron haem chromophore that absorbs in the near UV and visible spectral region (Ruckpaul et al, 1989). The most intense absorbance band is the Soret band, and this is due to the transfer of an electron from the iron to the haem group. Upon the addition of an electron and CO the Soret band shifts to approximately 450nm, since CO binds to the iron (II) form of the protein. This complex has been used to determine the concentration of P450s for many decades (Omura and Sato, 1964a; Omura and Sato, 1964b are constantly referenced in the literature). The formation of this complex without the appearance of a P420 peak also indicates that the P450 is potentially fully active, since this means that all the P450 molecules are capable of binding CO and therefore O₂, the precursor for forming the active oxygen intermediate, as well. However, this experiment only indicates whether the iron has a free coordination site in the reduced state, it does not provide information as to whether a substrate, which is usually much larger than CO, can access the active site.

The wavelength at which the Soret band is maximal is dependent upon the electronic configuration of the iron. Iron (III), the resting oxidation state in P450s (Groves and Han, 1995), has five electrons in its d-orbitals and these can be arranged in one of two ways, which are called low and high spin states (Figure 3-2). A low spin state is where the least number of electrons are unpaired; this occurs when the energy gap (ΔE) between the e₉ and t₂g orbitals is larger than the repulsive forces that exists between the coupled electrons (Figure 3-2 a). When ΔE becomes sufficiently small,
Figure 3-2: d-orbitals splitting diagram in an iron complex.
(a) and (b) depict the low and high spin states, respectively, for an iron complex containing 5 d orbital electrons. $\Delta E_0$ and $\Delta E_1$ represent the energy differences between the $e_g$ and $t_{2g}$ states in the low and high spin states, respectively. In the high spin state the energy gap between the $e_g$ and $t_{2g}$ states is sufficiently smaller to make it energetically more favourable for the electrons to uncouple and occupy the $e_g$ orbitals.

The electrons will uncouple and occupy the $e_g$ orbitals, even though they are at a higher energy, because the repulsive force between the electrons is greater. In this case, where there is the greatest number of electrons unpaired, the system is in a high spin state (Figure 3-2 b) (Shriver et al., 1994). It is due to the presence of unpaired electrons that iron is a paramagnetic species and can be used as a probe in the NMR experiments.

In the absence of a substrate P450 101, a model bacterial P450, has been found to be
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Figure 3-3: UV Visible spectrum (300-500nm) of P450 101.

a) Low spin form. P450 101 diluted into 50 mM Tris pH 7.5.
b) High spin form. P450 101 diluted into 50 mM Tris, pH 7.5, 1 mM camphor, 200 mM KCl, respectively (Westlake, 1997, unpublished results).

entirely low spin, as determined by optical (Sligar, 1976) and EPR (Lange et al., 1980) spectrosopies. This is due to the presence of a water ligand (Poulos et al, 1986; Raag, 1989) coordinated to the iron in the sixth position, i.e. trans to the thiolate group. Upon substrate binding to P450 101, the water ligand is expelled to give a five coordinate iron, as demonstrated by the x-ray crystal structure of this species (Poulos et al, 1987), and a high spin state is formed (Martinis et al, 1991).

The absorbance bands due to low and high spin states of P450s are quite different in terms of peak maxima and shape (Figure 3-3). P450 101 has absorbance maxima of 418 and 390nm for the low and high spin states, respectively, and most other P450s have very similar maxima, differing by only one or two nanometer. These differences between the two spin states have been utilised to investigate substrate binding, as can be seen in Chapters Four and Five.
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However, not only substrates give rise to spin state changes. Further analysis of the spin state of P450 101, demonstrated that the substrate free, low spin, enzyme was in a dynamic equilibrium with the high spin form, and this could be affected by temperature, ionic strength and pH, although not to the same extent as by substrate binding (Lange et al., 1980; Sligar, 1976; Lange et al., 1979; Lange et al., 1977). In addition, due to the large differences in active site structure, P450s have been found in low, e.g. P450 2C9 (Poli-Scaife et al, 1997), high, e.g. human P450 1A2 (Regal and Nelson, 2000), and mixed spin states, i.e. a combination of both low and high spin states, e.g. P450 2B1 (Hanna et al, 1998).

By characterizing the Soret band of purified P450 3A4 it should be possible to assess whether a substrate/inhibitor is present that could impede the access of the substrate under investigation, since P450 3A4 has been produced in a "mainly low spin state" (Guengerich et al, 1986) in the past.

3.1.3.2 P450 3A4 fluorescence turnover assay with 7-benzyloxyquinoline

In order to assess the catalytic activity of the P450 3A4 preparations a quick method, i.e. one that would avoid the requirement of an HPLC assay, was required. By adapting a protocol developed by the company Gentest (www.gentest.com), an assay of this kind was produced. This work was based on studies done by Mayer et al (Mayer et al., 1990) who first synthesized 7-benzyloxyquinoline (7-BQ), and demonstrated it was a substrate for the P450s present in rat liver microsomes.

The assay depends on the fact that 7-BQ is oxidized by P450 3A4 on the benzylic carbon, which gives rise to the highly fluorescent compound 7-hydroxyquinoline (7-HQ) Figure 3-4). As a result, the reaction can be observed directly by monitoring the increase in fluorescence of this molecule (Figure 3-5). This is instead of having to take multiple aliquots at different time points, quench the reaction and then determine the amount of product formed using HPLC.
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Figure 3-4: Reaction scheme of 7-BQ oxidation by P450 3A4.
The red arrow indicates where 7-BQ is oxidised.

Figure 3-5: Example of a kinetic trace obtained for the 7-BQ turnover assay.
The part of the kinetic trace marked a) is where the reaction is initiated with cumene hydroperoxide.
In the Gentest assay, the whole P450 catalytic system is present i.e. CPR, microsomal P450 3A4, and an NADPH regenerating system. However, in the present study, no phospholipids will be present in the buffers that will be used for the optical and NMR substrate binding experiments. Thus, in order to get a more accurate picture of the catalytic activity of the P450 3A4 samples in the absence of phospholipids an alternative system was designed. Since P450 and CPR only interact effectively in the presence of phospholipids (Imaoka et al, 1992), an organic hydroperoxide, i.e. cumene hydroperoxide (Figure 3-6), was used instead to form the active intermediate. Organic hydroperoxides can directly provide the oxygen and two electrons required to produce this highly reactive intermediate; this is called the peroxide shunt mechanism (Hrycay et al, 1976).
It should be noted, however, that this assay was developed to check that the purified P450 3A4 samples could turnover a substrate, thus demonstrating that substrates could access the active site of the protein. It was not designed to determine the kinetic parameters, \( i.e. \) \( K_m \) and \( k_{cat} \), of the reaction.

### 3.2 Results and discussion of purification

#### 3.2.1 Flowchart of P450 3A4 preparation

The following flowchart gives an outline of the procedure for obtaining recombinant cytochrome P450 3A4 (Figure 3-7). As can be seen, steps I-III (tan) are common to both method A (pale blue) and B (light green). The results discussed here are for the main routes adopted to obtain P450 3A4 for characterisation, optical binding and NMR experiments. However, many minor alterations to the purification method were also made in order to obtain an NMR active sample of P450 3A4 but these samples were not so well characterised and are discussed in Appendix C.

![Flowchart Scheme of P450 3A4 Preparation](image-url)

*Figure 3-7: Flowchart scheme of P450 3A4 preparation.*
This part of the chapter will be divided into the steps described in the flowchart. First, the results from methods A1 and A2 will be presented so that the effects of E911 and CHAPS on the purification can be directly compared. After this, the results from method B will be discussed.

Method A1 was the protocol that was followed initially, steps I-IV with E911 as the detergent, since this was the route used in our laboratory in the past to prepare P450 3A4 for the NMR experiments (Modi, 1996, unpublished results).

3.2.2 Step I

JM109 cells containing the pCW vector were grown in LB media overnight at 37°C. This culture was then used to inoculate (1 in 100) a TB medium contained within a 2L conical flask. At this stage, various different volumes (250, 400 and 500mL) of TB media were tried to establish whether aeration was a critical factor to cell growth. It was found that all gave approximately the same yield of 18-22g wet cell weight per litre of culture and so 500mL cultures were used from then on.

In an attempt to monitor P450 3A4 expression using SDS-PAGE, TB cultures that had been induced with IPTG (0.2, 0.5 and 1 mM) were taken through to the ultracentrifugation step, whereupon samples were taken for analysis. Samples were not taken just after harvesting, since these would contain not only folded membrane-bound P450 3A4, but also inclusion bodies (unfolded P450 3A4 that will not be recovered).

The following gel, Figure 3-8, demonstrated that it was not possible to monitor P450 3A4 expression using SDS-PAGE, since there are two bands that are very close to the band of authentic P450 3A4, one slightly above and the other just below. Therefore, it is likely that P450 3A4 is being expressed at a similar or lower concentration to other E.coli proteins. It could also be seen that higher concentrations of IPTG (0.5 and 1 mM) or longer growth periods did not dramatically affect either of these bands. As a result, 0.2 mM IPTG was used and a 24 hour growth period.
Figure 3-8: 10\% SDS-PAGE gel showing protein expression under various conditions in E.coli JM109 cells containing the P450 3A4 gene.

The lanes in the gel correspond to:
1: High weight markers (36-205 kDa)
2: Pre-stained markers (25, 33, 48, 83, 175 kDa)
3: Purified P450 3A4 control (3 µg P450)
4: E.coli cells containing the P450 3A4 gene grown for 24 hours and induced with 0.2 mM IPTG.
5: E.coli cells containing the P450 3A4 gene grown for 24 hours and induced with 0.5 mM IPTG.
6: E.coli cells containing the P450 3A4 gene grown for 24 hours and induced with 1 mM IPTG.
7: E.coli cells containing the P450 3A4 gene grown for 48 hours and induced with 0.2 mM IPTG.

10 µL of each sample was loaded into each lane.

In retrospect, it would have been more rigorous to perform this experiment with the correct control, i.e. E.coli cells without the pCW plasmid containing the P450 3A4 gene grown under the same conditions, since this would have provided a clearer comparison.
Sphaeroplasts were prepared as described in section 2.2.1.3 and then frozen at -20°C.

3.2.3 Step II

The sphaeroplasts were thawed and prepared for solubilisation as described in section 2.2.1.4. As the sphaeroplast samples thawed, they started to lyse and liberate a large amount of DNA that produced a highly viscous suspension, which was very difficult to resuspend in buffer A. Thus, DNase I, (RNase free; 10 units/ml) and 5 mM magnesium chloride was added to the suspension, which made it possible to generate a homogeneous solution within half an hour. When the solution was sonicated it became noticeably darker red/brown in colour and less viscous. No further change in the colour, possibly indicative of the amount of P450 being released from the membrane, or the viscosity of the solution was observed after 4 cycles of sonication and it was stopped at this point. P450 concentration determination at this stage, see Figure 3-9, gave rise to peaks at 420 and 450nm in the visible spectrum. With hindsight, the sonication step should have been optimised on the greatest recovery of P450 rather than purely on the colour or viscosity. Although P450 is denatured to give P420, it is more likely that this 420nm absorbance is due to other cytochromes, expressed by the host cells, which can coordinate the CO. This could have been verified if E.coli cells without the P450 3A4 gene had been grown and purified in parallel to those that did contain this gene.

P450 3A4 was then solubilised from this solution by using a detergent (either E911 or CHAPS).

3.2.3.1 Solubilisation using E911 (Method A1)

There is little in the literature pertaining to E911’s surfactant properties (i.e. its CMC or CMT) but it does contain very similar structural features to Triton N-101, whose detergent properties have been well characterised (Dawson et al., 1986; Neugebauer, 1992) (Figure 3-10).

Triton N-101 has a CMC of 85 μM or 0.005% w/v in aqueous solution at 25°C (Dawson et al., 1986). The concentration of E911 used to solubilise P450 3A4 from
0.16  
0.14  
0.12  
0.10  
0.08  
0.06  
0.04  
0.02  
0.00  
-0.02  

Wavelength (nm)

350 400 450 500

Absorbance

Cytochrome impurities

P450 3A4

Figure 3-9: Fe$^{2+}$-CO vs Fe$^{2+}$ difference spectrum after sonication.
The sonicated solution was diluted four fold with 0.1 M KPB, 10% v/v glycerol, 0.1% E911 pH 7.5 (buffer Z) and reduced with sodium dithionite. This solution was split between two cuvettes and a baseline was taken. The sample cuvette had carbon monoxide bubbled through it for about 30 seconds after which an optical difference spectrum was taken.

its membrane was 0.1% w/v, and so assuming the CMC for E911 is similar as Triton N-101, then this concentration should be sufficient to produce E911 micelles and thus solubilise the P450 from the membrane. However, it was found that another group (Gillam et al., 1993) use concentrations as high as 0.5% w/v E911 for their extractions, although this may just be because even higher concentrations (1% w/v) were used originally (Sato et al, 1973). In order to ensure that sufficient E911 was being used a trial was performed to see how P450 extraction was affected by E911 concentration. This was achieved by taking aliquots from the sonicated solution, adding 0.01, 0.02, 0.05, 0.1, 0.2, and 0.4% w/v E911 and then after ultracentrifugation determining the amount of P450 present in solution. This trial demonstrated that (see Table 3-1), the majority of the P450 was extracted with 0.05% E911, but 0.1% w/v was used to ensure maximum recovery of the protein from the membrane, which was 199 +/- 29 nmol per litre of culture.
Figure 3-10: Comparison of the chemical structures of E911 and Triton N-101.

i) E911 - structure obtained from Karlan Research Products Corporation.

ii) Triton N-101 - structure obtained from Dawson et al., 1986.

The relative proportions of the different numbers of ethylene oxide side chain for Triton N-101 have not been reported.

<table>
<thead>
<tr>
<th>% w/v E911</th>
<th>[P450]/μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.06 +/- 0.016</td>
</tr>
<tr>
<td>0.02</td>
<td>0.176 +/- 0.022</td>
</tr>
<tr>
<td>0.05</td>
<td>0.691 +/- 0.029</td>
</tr>
<tr>
<td>0.1</td>
<td>0.735 +/- 0.06</td>
</tr>
<tr>
<td>0.2</td>
<td>0.661 +/- 0.023</td>
</tr>
<tr>
<td>0.4</td>
<td>0.766 +/- 0.023</td>
</tr>
</tbody>
</table>

Table 3-1: P450 solubilisation trial using E911.

Aliquots of sonicated sphaeroplast were diluted into buffer A containing the following final % w/v concentrations of E911: 0.01, 0.02, 0.05, 0.1, 0.2 and 0.04%. These samples were stirred for 1 hour at 4°C, after which time they were ultracentrifuged for 1 hour and then diluted two fold into buffer Z so that their P450 concentrations could be determined using the optical method described in section 2.2.2. Values are the means +/- standard deviation based on three experiments.
Figure 3-11: Fe^{2+}-CO vs Fe^{3+} difference spectra of solubilisation trial of P450 3A4 using E911.

(a) and (b) show the effect of 0.01-0.05% and 0.1-0.4 % E911, respectively, on the amount of P450 solubilised from the E.coli membrane. All samples were diluted two fold into buffer Z and reduced using sodium dithionite. Each sample was split between two cuvettes and a baseline was taken. The sample cuvette then had carbon monoxide bubbled through it for about 30 seconds after which an optical difference spectrum was taken.

It was found that, at low concentrations of detergent, there was very little P450 solubilised and some P420 was present (Figure 3-11 a). However, as the detergent concentration was increased from 0.02 to 0.05% w/v a dramatic increase in the recovered P450 occurs. Above 0.05% almost equivalent amounts of P450 were removed from the membrane, even when 8 times as much detergent was used (Figure 3-11 b). If detergent micellisation is required for protein solubilisation then the actual CMC of E911, in this system, must be between 0.02 and 0.05%, which is much higher than the reported value. This could be due to the high glycerol concentration, since this compound has been reported to weaken interactions between proteins and hydrophobic interaction columns (Shukla et al., 2002); this suggests that it is able to assist the solubilisation of a hydrophobic molecule in some way.
Chapter Three

Figure 3-12: Fe2+-CO vs Fe2+ difference spectrum of cell pellet after ultracentrifugation.

The cell pellet was resuspended in buffer Z, after which the solution was reduced using sodium dithionite. This solution was split between two cuvettes and a baseline was taken. The sample cuvette then had carbon monoxide bubbled through it for about 30 seconds after which an optical difference spectrum was taken.

Figure 3-13: Chemical structure of CHAPS.
Structure obtained from Dawson et al., 1986.

In addition to the supernatant, the pellet was also checked to see if it had any remaining P450. It can be seen from Figure 3-12 that there was an insignificant amount of P450 in the cell pellet and the majority of the sample gave rise to a 420nm peak, assumed to be other cytochrome impurities.
Figure 3-14: Comparison of solubilisation of P450 3A4 using either E911 or CHAPS.

Either 0.1% w/v E911 or 10 mM CHAPS were added to aliquots of sonicated sphaeroplasts. These samples were stirred for 1 hour at 4°C and then ultracentrifuged. Both supernatants were diluted four fold into buffer Z after which the samples were reduced using sodium dithionite. These samples were split between two cuvettes and a baseline was taken. The sample cuvette then had carbon monoxide bubbled through it for about 30 seconds after which an optical difference spectrum was taken.

3.2.3.2 Solubilisation using CHAPS (Method A2)

CHAPS is a bile acid derivative detergent (Figure 3-13), which has a CMC between 8-11 mM (Hjelmeland et al., 1983; Stark et al., 1984) in an aqueous solution at 20°C. It has been used by other groups (Domanski et al., 1998; Hosea et al., 2000) at a concentration of 10 mM or above to solubilise P450 3A4. In this study, 10 mM CHAPS was used, and found to solubilise 185 +/- 25 nmol P450 3A4 per litre of cell culture, which was nearly as effective as 0.1% E911 cf. 199 +/- 29 nmol (Figure 3-14). These yields are similar to those that have been reported in the past (Pritchard et al., 1997).
3.2.4 Step III

After solubilisation the protein was purified using a nickel affinity column in the presence of either 0.1% w/v E911 or 10 mM CHAPS, since the construct used in these experiments had a six histidine tag on the C-terminus (appendix A).

3.2.4.1 P450 3A4 solubilised with E911 (Method A1)

The nickel affinity column purification was monitored at 280nm and the chromatogram produced is shown in Figure 3-15 (i).

Fractions A-D were collected and analysed by SDS-PAGE, the results of which can be seen in Figure 3-15 (ii). The gel of the purification demonstrates how effective the affinity column is at removing contaminating proteins and at concentrating the protein. The resulting P450 3A4 solution does, however, still has some higher molecular weight impurities, their closest markers are 84, 116 and 205 kDa, but these are at a lower concentration.

The fractions collected were all a red/brown colour, which suggests that they may contain P450 3A4. However, as pointed out in step II, this colour is due mainly to the presence of cytochromes produced by the E.coli, and thus each fraction was analysed for P450 content, see Figure 3-16. The flow through solution was found to contain about 25% of the total P450 loaded. This could be because the P450 was interacting with an impurity in such a way as to prevent the histidine tag from being available to bind to the nickel affinity column. However, this stage was not investigated further since sufficient protein was being produced to perform the characterisation, optical binding and NMR relaxation experiments.

The fractions eluted with 30 mM imidazole were also found to contain P450. However, these fractions also contained a large quantity of P420, see Figure 3-16, and so were not analysed further. Those fractions eluted with 60 mM imidazole contained an insignificant amount of P450 and so were not analysed further.
Figure 3-15: Chromatogram and 10% SDS-PAGE gel of nickel affinity column purification (E911) measured at 280nm.

In the chromatogram i), A (4) is the flow through and B (5), C (6) and D (7) are the fractions eluted with 30, 60 and 500 mM imidazole, respectively. The number inside the brackets denotes the samples' lane number in the gel. The lanes in the gel (ii) are as follows:

1: High Weight Markers (36-205 kDa)
2: Purified P450 3A4 control
3: Ultra-centrifuged supernatant
4: Flow through off nickel affinity column.
5: 30 mM Imidazole wash
6: 60 mM Imidazole wash
7: 500 mM imidazole elute (3 µg P450).

10 µL of each sample was loaded into each lane.
Figure 3-16: Fe$^{2+}$-CO vs Fe$^2$ difference spectra of the fractions off the nickel affinity column.

Each fraction was diluted four fold with buffer Z and reduced with sodium dithionite. Each diluted fraction was then split between two cuvettes and a baseline was taken. The sample cuvette had carbon monoxide bubbled through it for about 30 seconds after which an optical difference spectrum was taken.

P450 3A4 was then eluted in a tight band off the column using 500 mM imidazole; the usual yield was approximately 130 nmol per litre of culture. Initially, after this stage, the protein was dialysed back into buffer AE. This resulted in the protein precipitating as could be seen by a sloping baseline in the UV-Vis trace (data not shown) and by the fact that a red pellet was formed upon centrifuging the protein solution at 13,000 rpm in a microcentrifuge. It was found that using a buffer containing EDTA, DTT and no salt reduced precipitation to a minimum. As a result, the eluant was usually diluted three times by QE buffer and then dialysed against it three times, at a ratio of 1:10, over a 40-hour period.

DTT is a standard reagent in the preparation of proteins and is known to help prevent disulphide bond formation by preventing oxidation of the protein (Thomas and McNamee, 1990; Chambers, 1993); indeed, it is essential for obtaining monomeric P450$_{cam}$ (Nickerson and Wong, 1997). The chelating agent EDTA was also added in
order to i) inhibit any remaining proteases which require metal ions and ii) remove any paramagnetic ions from the protein solution, as these would complicate the NMR experiments.

3.2.4.2 P450 3A4 solubilised using CHAPS

The chromatogram of the affinity purification measured at 280nm was identical to that obtained when using E911, see Figure 3-16 a), and so is not included. An SDS-PAGE gel illustrating the high purity of P450 3A4 is shown in Figure 3-17. This time, however, only one higher molecular weight impurity is observed (closest marker 116 kDa).

It was observed that the flow through, 30 and 60 mM imidazole wash fractions were red/brown in colour and so these were checked for the presence of P450. The flow through was found to contain mainly the species that absorbs at 420nm and very little P450. This was different to what was observed when in the presence of E911, and could suggest that fewer impurities are solubilised with CHAPS and so more P450 3A4 molecules are free to bind to the metal affinity column. The 30 and 60 mM imidazole wash fractions produced very similar Fe^{2+}-CO vs Fe^{2+} difference spectra to those observed in Figure 3-16.

P450 3A4 was then eluted using imidazole (500 mM) and dialysed into QC buffer, the yield at this stage was approximately 185 nmol per litre. The CHAPS concentration was reduced at this stage to 2 mM, since it was found that when this detergent is present at a concentration of 10 mM it generates a high spin P450 species, which suggests that it binds to the active site of P450 3A4, see section 3.3.1.2, Figure 3-24.
Figure 3-17: 10% SDS-PAGE gel of nickel affinity purification (CHAPS).
The lanes in the gel correspond to:
1: High Weight Markers (36-205 kDa).
2: P450 3A4 control.
3: Ultra-centrifuged supernatant.
4: Flow through.
5 and 6: 30 mM imidazole wash.
7: 60 mM imidazole wash.
8: 500 mM imidazole elute fraction (3µg P450).
10 µL of each sample was loaded into each lane.

3.2.5 Step IV

The conductivity of the protein solution was found to be almost identical to the
dialysis buffer (either QE or QC) after three changes, which demonstrated that the
buffer exchange step had gone to completion and thus that the sample could be loaded
onto the HiTrap Q column.

3.2.5.1 P450 3A4 solubilised with E911 (Method A1)

Figure 3-18 i) and ii) demonstrates how the majority of P450 3A4 (solubilised with
E911) flows through the HiTrap Q column. However, some is retained, as is
evidenced by the fact that a broad light red band appears at the top of the column.
Most of the retained P450 3A4 elutes as a sharp band after three column volumes of
Figure 3-18: Chromatogram and 10% SDS-PAGE gel of HiTrap Q column purification (E911).

In the chromatogram i), A (4) is the flow through and B (5), C (6), D (7), E (8) and F (9) are the fractions eluted with 50, 100, 200, 250 and 500 mM KCl, respectively. The number inside the brackets denotes the samples' lane number in the gel. The lanes in the gel (ii) are as follows:

1: P450 3A4 control
2: Low Weight Markers (66, 45, 24 and 14.2 kDa).
3: Before HiTrap Q (2μg P450).
4: Flow through off Hi Trap Q column.
5: 50 mM KCl wash.
6: 100 mM KCl wash.
7: 200 mM KCl wash.
8: 250 mM KCl wash.
9: 500 mM KCl wash.
10: Purified P450 3A4 control

10 μL of each sample was loaded into each lane.
50 mM KCl, but then the remainder is eluted with 200 and 250 mM salt concentrations. No colour was observed in the fractions obtained at the highest salt concentrations used, which suggested that there was little or no P450 3A4 present. Figure 3-18 (ii) also shows that the P450 3A4 sample produced is slightly more pure, since higher and lower weight impurities have bound more tightly to the column and thus been removed.

Pritchard et al (Pritchard et al., 1997) reports that P450 3A4 flowed through a Hi Trap Q column when using essentially identical buffer conditions. This could be because they did not observe the small fraction that bound, which is possible if they only expressed a small amount and employed a linear gradient to elute impurities from the column. Alternatively, the protein could be close to its pi at this pH, and therefore if the pH, in this study, were slightly higher than 7.5 this could cause it to bind weakly to the HiTrap Q column. However, since sufficient quantities were collected for characterisation and substrate binding studies this step was not analysed further.

The flow through and KCl (50 mM) fractions were collected, since they contained the greatest amounts of purified P450, and concentrated using a Centriprep YM-30 to at least 50 μM, the yield of these two fractions was approximately 95 nmol per litre of culture. These fractions were then prepared for characterisation, as described in section 3.3.

3.2.5.2 P450 3A4 solubilised with CHAPS (Method A2)

When P450 3A4 (solubilised with CHAPS), was applied to an HiTrap Q column it was found to bind (Figure 3-19 a and b) and then elute at 50, 100 and 200 mM KCl. It could also be seen from the gel (Figure 3-19 b) that this step yielded slightly purer P450 3A4, since the higher weight molecular impurities bound more strongly to the HiTrap Q column, and so were eluted at a much higher ionic strength.

The observation that P450 3A4 binds to an anion exchange column is contrary to what was expected since the majority of P450 3A4 flows through the HiTrap Q column when in the presence of 0.1% v/v E911. E911 is non-ionic and so is unlikely to
Figure 3-19: Chromatogram and 10% SDS-PAGE gel of HiTrap Q column purification of P450 3A4 using 2 mM CHAPS.

In the chromatogram (i), A (3), B (4), C (5), and D (6) are the fractions eluted with 50, 100, 200 and 500 mM KCl, respectively. The number inside the brackets denotes the samples’ lane number in the gel. The lanes in the gel (ii) are as follows:

1: High Weight Markers (36-205 kDa).
2: 3A4 before HiTrap Q (4μg P450).
3: 50 mM KCl wash (3μg P450, concentrated before loading).
4: 100 mM KCl wash (5μg P450, concentrated before loading).
5: 200 mM KCl wash.
6: 500 mM KCl wash.

10 μL of each sample was loaded into each lane.
weaken ionic interactions between the protein and the anion exchange column; therefore, it is reasonable to assume that P450 3A4 does not have a significant net negative charge under these conditions. In addition, Hosea et al (Hosea et al., 2000) recently reported that P450 3A4 does not bind to a diethylaminoethyl (DEAE) resin at pH 7.4 when in the presence of 20 mM CHAPS. Although the protein would not be expected to bind to a DEAE resin as tightly as an HiTrap Q column, since DEAE is a weak anion exchange resin, if the protein had a net negative charge then some interaction would be expected. However, if there was only a weak net negative charge it is possible that 20 mM CHAPS could increase the ionic strength of the medium sufficiently to prevent an interaction from occurring.

One possible explanation for why P450 3A4 binds to an HiTrap Q column could be because of the effect of low concentrations of CHAPS on the protein. If P450 3A4 has a net negative charge due to the presence of CHAPS, which could occur because of exposed sulphonate groups or due to masking of positive charge, and the concentration of CHAPS is low enough not to effect the ionic strength of the medium then this complex would be able to interact with the HiTrap Q column. However, P450 3A4 does not elute at one ionic strength, which would suggest that, either each protein molecule does not have the same number of CHAPS molecules bound, or the protein exists as a combination of differently sized aggregates. The fact that the presence of 20 mM CHAPS actually prevents binding to an anion exchange column would suggest that the protein surface can be saturated by this detergent, and that the negative charge generated by it is only weak, since the medium's ionic strength would not be significantly increased by this amount of CHAPS. However, it has been shown that 20 mM CHAPS can disaggregate P450 2B4 to a monomer (Viner et al., 1995). If a similar result occurred with P450 3A4, then Hosea et al (Hosea et al., 2000) may not have observed an interaction with a DEAE resin because the protein was in a less aggregated state, and so had a much smaller net negative charge. Therefore, in this study, P450 3A4 may have bound to the HiTrap Q column and eluted at different ionic strengths because it was in a combination of oligomeric states, due to insufficient CHAPS being present to keep it monomeric.

The fractions eluted with 50 and 100 mM KCl were collected, since they contained the greatest amounts of P450 at the highest purity, and concentrated using a
Centriprep YM-30 to at least 50µM, the yield of these two fractions was approximately 80 nmol per litre of culture. These samples were then handled as described in section 3.3 so that they could be characterised. The fraction eluted with 200 mM KCl was not collected as it still contained a higher molecular weight impurity.

3.2.6 Step V

This step differentiates method A2 from method B. Method A2 has shown that an ion-exchange step provides only a slight improvement in purity, but also has highlighted that the protein may exist as aggregates. Size-exclusion chromatography was used because it offered the potential to:

a) Remove the higher weight impurities and imidazole that is still present after the affinity step within 2-3 hours; cf. extensive dialysis takes 40 hours.

b) Buffer exchange the protein into a buffer similar to that used in NMR so that optical binding experiments can be performed without further steps.

c) Ascertain the aggregation state of the protein.

The HiLoad 26/60 Superdex 200 column that was employed had been previously calibrated (Haldane, 2001, unpublished results) using a set of molecular weight standards obtained from Sigma-Aldrich (Table 3-2). The buffer used for the calibration was 50 mM Tris-HCl, 200 mM KCl, 5% v/v glycerol, and the buffer employed in the purification was 100 mM KPB, 150 mM KCl, 5% v/v glycerol, 1 mM DTT, 1 mM EDTA and 2 or 0.5 mM CHAPS pH 7.5 (Buffer S). The fact that it was calibrated in a different buffer to that being used in this study should not matter, since the intrinsic pore sizes of the beads in the column should not change. However, if one of the buffer constituents binds to the interior of the beads, or is able to reduce the diameter of the beads' pores in some other way, then this could prevent the column from being able to resolve larger hydrodynamic diameter molecules. The only compound likely to be able to achieve this is CHAPS as it can form large micelles, but practically this does not seem to be the case as it has been shown that when in the absence or presence of CHAPS (16 mM) a sample of P450 2B4 elutes in exactly the same volume from an Ultragel AcA 44 size (Viner et al, 1995).
### Table 3-2: Calibration data for HiLoad 26/60 Superdex 200 (Haldane, 2000).

Protein standards were loaded as a mixture in 2mL. The void volume, $V_0$, was determined to be 115mL using blue dextran.

<table>
<thead>
<tr>
<th>Protein Standard</th>
<th>MW (kDa)</th>
<th>Approximate concentration (mg/mL)</th>
<th>$V_e$ (mL)</th>
<th>$w_h$ (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonic anhydrase</td>
<td>29</td>
<td>2</td>
<td>234</td>
<td>8</td>
</tr>
<tr>
<td>Albumin</td>
<td>66</td>
<td>7</td>
<td>199</td>
<td>8</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>150</td>
<td>3</td>
<td>176</td>
<td>8</td>
</tr>
<tr>
<td>β-Amylase</td>
<td>200</td>
<td>2</td>
<td>161</td>
<td>8</td>
</tr>
<tr>
<td>Apoferritin</td>
<td>443</td>
<td>7</td>
<td>142</td>
<td>7</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>669</td>
<td>5</td>
<td>126</td>
<td>8</td>
</tr>
</tbody>
</table>

In this method, as soon as P450 3A4 was eluted from the HiTrap affinity column it was loaded onto the HiLoad 26/60 Superdex 200 column. A typical chromatogram obtained from this step can be seen in Figure 3-20 i. The four main fractions eluted were labelled A-D, A being the first fraction collected and therefore containing the largest molecular weight proteins or aggregates of proteins.

Fractions A-C were collected, concentrated using a Centriprep YM-30, and analysed using SDS-PAGE (Figure 3-20 ii). Fraction D was not collected because the time at which it eluted from the S200 column suggested that its molecular weight was around 2 kDa, which is far too low for P450 3A4.

Fraction A elutes in the void volume, which corresponds to a species with an average molecular weight size of greater than 700 kDa, but unexpectedly SDS-PAGE shows that it contains mainly P450 3A4, which suggests that it is highly aggregated. As a result of this and the fact that this fraction contained very little P450 3A4 it was not analysed further. Fraction C was found to have an average molecular weight of 50
Figure 3-20: Chromatogram and 10% SDS-PAGE gel of a size-exclusion purification using an S200 HiLoad 26/60 using buffer S containing 0.5 mM CHAPS.

In the chromatogram (i), A (4), B (5), C (6), and D correspond to average molecular weight sizes of > 700, 300, 46 and 1 kDa. The number inside the brackets denotes the samples' lane number in the gel. The red arrow indicates where monomer P450 3A4 would be expected to elute. The lanes in the gel ii) are as follows:

1: High Weight Markers (36-205 kDa).
2: Purified P450 3A4 control (5μg P450)
3: Elute fraction from nickel affinity column.
4: Fraction A (concentrated).
5: Fraction B (5μg P450).
6: Fraction C (concentrated).

10 μL of each sample was loaded into each lane.
kDa, which might have suggested monomer P450 3A4. The SDS-PAGE, however, shows that this sample is mainly a lower weight molecular impurity, which suggests that under native conditions it is highly aggregated, and thus this fraction was not investigated further.

Fraction B was found to contain the majority of P450 3A4, and the yield was approximately 130 nmol per litre of culture. The average molecular weight of fraction B was found to be either ~220 or 300 kDa when in the presence of either 2 mM or 0.5 mM CHAPS, respectively. In the calibration, the peak width at half height was found to be 8mL, whereas, in these experiments fraction B was normally found to be at least 20mL. This indicates that P450 3A4 exists in a range of oligomeric states that overlap with each other, which leads to a very broad peak. It can also be seen from Figure 3-20 ii) that the size-exclusion column removes some of the contaminating proteins from the P450 3A4 sample, but three higher molecular weight impurities remain.

The only report that refers to the aggregation state of P450 3A4 is by Benes M. et al. (Benes et al., 2001). By using fluorescence correlation spectroscopy, they found that when the protein's concentration was above 1 μM the diffusion time of a coumarin 6/P450 3A4 complex increased by two fold. They suggested that this was due to P450 3A4 aggregating, and estimated its size to be that of a hexamer, though they point out that the accuracy of this estimate is dependent on the shape of the protein, which was assumed to be globular. P450s have been described as a flat, triangular shaped molecule (Poulos et al., 1995), based on the crystal structures of P450 101, 102 (haem domain) and 107, thus this assumption is an appropriate one, since P450 3A4 is unlikely to be rod shaped. In this study, the average oligomeric state of P450 3A4 was either a tetramer or pentamer when in the presence of either 2 or 0.5 mM CHAPS, respectively. However, the estimate derived from size-exclusion chromatography is also very much dependent on the shape of the protein molecule, which was again assumed to be globular. It is also worth noting that this estimate does not take into account bound CHAPS molecules, which would also affect the size and shape of the P450 aggregate.
The aggregation of solubilised and purified microsomal P450s is, however, extremely common. P450 1A2, 2B4 (Viner et al., 1995), a truncated form of 2D6 (Kempf et al., 1995) and 2E1 (Pernecky et al., 1995) have all been purified to homogeneity and found to be in an aggregated state. The formation of monomers could only be brought about for P450 1A2 and 2B4 by the addition of high concentrations of CHAPS (20 mM) (Dean and Gray, 1982; Wagner et al., 1987). P450 2D6 was truncated by removing 25 amino acid residues from the N-terminus in an attempt to make the protein more soluble. Kempf et al (Kempf et al., 1995) seemed to have achieved this aim, since the protein was found in the cytosol after the cells had been disrupted instead of the membrane. However, they then discovered that 50% of the protein was in a highly aggregated state and that it could only be made monomeric by the addition of 1% w/v nonaethyleneglycol monododecyl ether, a non-ionic detergent that is often used to try to crystallise membrane proteins. P450 2E1 could not be made into a monomer even in the presence of cholate at 1% w/v. However, a truncated form of P450 2E1 where residues 3-29 of the N-terminal region were removed was found to be a monomer when 0.5% sodium cholate was used. Thus, it can be seen that mammalian P450s are usually aggregated when removed from the membrane, unless detergents are added to sufficiently high concentrations.

3.2.7 N-terminal sequencing

In order to check that the samples of P450 3A4 generated were unmodified, they were investigated using N-terminal sequencing.

The construct used in these experiments was modified at the N-terminus with an ompA+2 leader sequence (ompA =MKKTAIAIAV ALAGFATVAQ A +2 =AP). This leader sequence is a bacterial signal peptide that directs the polypeptide chain to the bacterial inner membrane. During translocation of the polypeptide chain across the inner membrane, the signal peptide is removed and rapidly degraded to leave the native protein. Interestingly, it was found that the presence of the two additional amino acids, alanine and proline, are crucial for the cleavage of the ompA leader sequence (T. Friedberg, Personal Communication).
The N-terminal sequencing gave the following result for purified P450 3A4: Ala Pro Met Ala Leu Ile Pro Asp Leu Ala. This result is identical to the N-terminal sequence of P450 3A4, but with the additional amino acids alanine and proline present (Appendix A). Therefore, it was concluded that the correct protein had been expressed and purified.

3.3 Results and discussion of optical characterisation and catalytic activity of P450 3A4

P450 3A4 prepared by method A1 was buffer exchanged into a non-deuterated NMR buffer (100 mM KPB, 5% v/v glycerol, 0.1% w/v E911, 0.2 mM DTT pH 7.5) and was found to be visibly soluble up to 100 μM for at least a week at 4°C.

P450 3A4 prepared by method A2 and B, where 2 mM CHAPS was employed on the S200 column, was exchanged into the same non-deuterated buffer as described above, except with 2 mM CHAPS instead of E911. The protein was also found to be visibly soluble below 100 μM for at least a week at 4°C.

P450 3A4 prepared by method B with 0.5 mM CHAPS was exchanged, by Centriprep YM30, into Buffer S, but with 50 mM KPB instead of 100 mM, and was used purely for the optical binding experiments discussed in chapter Five. This protein was found to be as soluble as that prepared by B.

The protein samples were then characterised in the following ways:

1) The P450 concentration was determined by generating the reduced CO bound form of P450 3A4. This also made it possible to determine whether the sample contained P420, i.e. inactive protein (see section 3.3.1.1).

2) A UV/Visible spectrum (300-500nm) was taken, since this can give information about the local environment of the haem, i.e. whether any substrate/inhibitor is already bound (see section 3.3.1.2).

3) The samples catalytic activity towards the pro-fluorescent substrate 7-benzyloxyquinoline (7-BQ) was checked (see section 3.3.2).
Within each section, the data obtained from P450 3A4 produced by method A1 (flow through and 50 mM KCl fractions), method A2 (50 mM and 100 mM KCl fractions) and from method B (where 2 and 0.5 mM CHAPS was used) will be discussed. The samples names will be abbreviated to P450 3A4 (detergent, final column, a fraction description - if more than one was obtained during the purification), e.g. P450 3A4 (E911, Q, flow through) or (2 mM CHAPS, S200).

3.3.1 UV/Visible spectra of P450s and optical binding

3.3.1.1 P450 and P420 concentration determination

The concentration of P450 3A4 in the protein samples was determined in the standard way. In addition, since the cytochromes that give an absorbance peak at 420nm had been removed during the purification, the actual amount of P420, or inactive P450, could be investigated. The difference spectrum provides the best way to investigate whether P420 exists in a sample, since in the absolute spectrum a peak at 420nm could be due to either non-reduced P450 or inactive P450 that was generated by the addition of sodium dithionite or by the action of bubbling CO through the solution.

Typically, it was found that the samples of P450 3A4 contained very little or no P420, as judged visually (Figure 3-21).

3.3.1.2 Purified P450 3A4 absorbance spectra

In order to ascertain the resting spin state of the P450 3A4 samples their spectra were compared to mathematically generated simulations of P450 101 spectra at different states of high spin. The P450 101 simulated spectra were produced by a linear addition of 0% and 100% high spin P450 101 spectral data. By visually comparing the simulation of P450 101 with the closest 390nm/418nm value to a particular P450 3A4 sample, an estimate of the high spin content could be made.

The optical spectrum (300-500nM) of P450 3A4 (E911, Q, flow through) was compared to a 36% high spin simulation of P450 101 (Figure 3-22 i).
Figure 3-21: Typical Fe$^{2+}$-CO vs Fe$^{2+}$ difference spectrum observed for samples of P450 3A4 obtained by any of the methods.

Protein samples were usually diluted by 50 fold into buffer Z and then reduced using sodium dithionite. Each diluted sample was split between two cuvettes and a baseline was taken. The sample cuvette then had carbon monoxide bubbled through it for about 30 seconds after which an optical difference spectrum was taken.

Figure 3-22: UV Visible spectrum (300-500nm) of P450 3A4 (E911, Q, flow through) compared to P450 101.

(a) 1μM P450 3A4 (E911, Q, flow through) in 0.1 M KPB pH 7.5
(b) Simulation of 36% high spin P450 101 (Westlake, 1997, unpublished results).
(c) P450 101 0% high spin (same as above).
These absorbance spectra looked almost identical, except that P450 3A4 (E911, Q, flow through) has a slightly higher absorbance in the 300-370nm region, and thus it was assumed that this sample of P450 3A4 had approximately the same percentage of high spin present. In addition, a comparison between P450 3A4 (E911, Q, flow through) and 0% high spin P450 101 has been made in order to illustrate the fact that this sample of P450 was in a mixed spin state (Figure 3-22 ii). P450 3A4 (E911, Q, 50 mM KCl) produced a similar spectrum.

Since P450 3A4 has been produced in a "mainly low spin state" (Guengerich et al, 1986) in the past, this could indicate that in these preparations the protein already has a substrate or inhibitor bound. Alternatively, P450 3A4 could be affected by the phosphate or glycerol concentrations of the buffer, since Guengerich et al (Guengerich et al, 1986) used 5 mM KPB, 20%v/v glycerol pH 7.5.

By comparing the absorbance spectra of E911-purified P450 3A4 with a sample purified using CHAPS, it could be seen that E911 does cause a spin state change (Figure 3-23 i).

It was also interesting to note that when P450 3A4 (CHAPS, Q, 50 mM KCl) was compared to a 22% high spin simulation of P450 101 (Figure 3-23 ii), the spectra were similar in terms of their 390nm/418nm value but not in the 300-370nm region. This illustrates that P450 101 only gives an approximation of what the optical spectrum of P450 3A4 should look like, since these P450s probably have a different local haem environment that will affect the Soret absorbance band.

During the purification of P450 3A4 (CHAPS, Q), it was found that the presence of 10 mM CHAPS caused a large spectral shift towards high spin. By dialysing into the same buffer with less CHAPS (2 mM), the spin state could be shifted back to a more low spin state, which indicated that CHAPS was only weakly bound (Figure 3-24). A similar experiment could not be performed with E911, since this detergent forms very large micelles that are a comparable size to the P450 (Dawson et al., 1986).
Figure 3-23: UV Visible spectrum (300-500nm) of P450 3A4 (CHAPS, Q, 50 mM KCl) compared to P450 3A4 (E911, Q, flow through) and a simulation of 22% high spin P450 101.

a) 1 μM P450 3A4 (CHAPS, HiTrap Q, 50mM KCl) in 0.1 M KPB pH 7.5
b) 1 μM P450 3A4 (E911, HiTrap Q, flow through) in 0.1 M KPB pH 7.5

Figure 3-24: UV Visible spectrum of P450 3A4 (CHAPS, Q) (300-500nm).

a) P450 (CHAPS, HiTrap Q) dialysed into buffer QC containing 2mM CHAPS.
b) P450 (CHAPS, HiTrap Q) dialysed into buffer QC containing 10mM CHAPS.
These observations suggested that both E911 and CHAPS could bind to the active site of P450 3A4, and thus produce an optical spin state change. In fact, Hosea and Guengerich (Hosea and Guengerich, 1998) have reported that Triton N-101, which has a similar structure to E911, is oxidised by P450 3A4. They also reported that Triton N-101 had a $K_d$ of 7.2 $\mu$M with human liver microsomes, since it produced a titratable optical change upon addition, and that when it was present at 100 $\mu$M in human liver microsomes the binding of testosterone, a P450 3A4 selective substrate, was weakened. Thus, it is highly likely that E911 is a substrate/inhibitor for P450 3A4. There is no literature data on CHAPS being a P450 3A4 substrate, but since it has a steroidal structure (see Figure 3-13), it would seem reasonable that it could be. In the present study, a $K_d$ of 2.7 mM has been determined for this compound, see chapter Five.

The P450 3A4 (2 mM and 0.5 mM CHAPS, S200) absorbance spectra were almost identical to that produced by P450 3A4 (CHAPS, Q, 50 mM KCl) (data not shown).

### 3.3.2 Fluorescence turnover assay using 7-benzyloxyquinoline

By taking the gradient of the kinetic trace after the addition of cumene hydroperoxide, a set of initial rate data were obtained for each sample of P450 3A4 in terms of fluorescence units/min. By then using a calibration curve, which had been produced using an authentic standard of 7-HQ, it was possible to determine the initial rates in terms of [7-HQ] produced/min/nmol P450.

It was found that the samples of P450 3A4 produced by all of the methods were able to oxidise 7-BQ to 7-HQ. However, samples of P450 3A4 (CHAPS, Q or S200, all fractions) were able to turnover 7-BQ at least six times more rapidly than samples of P450 3A4 (E911, Q, both fractions) (Table 3-3). This could be because of the following reasons:

1) CHAPS increases the observed fluorescence of 7-HQ
2) E911 reduces the observed fluorescence of 7-HQ
3) E911 is inhibitory.
Table 3-3: A comparison of the initial rates of 7-HQ formation for P450 3A4 purified by the different methods.

1 μM P450 3A4 was mixed with 0.5 mM 7-BQ in 100 mM KPB, 5% v/v glycerol pH 8.0 and the reaction initiated with 1 mM cumene hydroperoxide. Initial rates were obtained from the first 10 seconds of data after reaction initiation and converted into rates of 7-HQ formation/nmol/min/nmol. P450 Values are the means +/- SD of three experiments.

<table>
<thead>
<tr>
<th>P450 3A4 sample</th>
<th>Rate of 7-HQ formation/nmol/min/nmol P450</th>
</tr>
</thead>
<tbody>
<tr>
<td>E911, Q, Flow through</td>
<td>0.34 +/- 0.05</td>
</tr>
<tr>
<td>E911, Q, 50 mM KCl</td>
<td>0.30 +/- 0.10</td>
</tr>
<tr>
<td>CHAPS, Q, 50 mM KCl</td>
<td>2.29 +/- 0.13</td>
</tr>
<tr>
<td>CHAPS, Q, 100 mM KCl</td>
<td>2.52 +/- 0.08</td>
</tr>
<tr>
<td>2 mM CHAPS, S200</td>
<td>2.50 +/- 0.20</td>
</tr>
<tr>
<td>0.5 mM CHAPS, S200</td>
<td>2.56 +/- 0.11</td>
</tr>
</tbody>
</table>

The effect of CHAPS on the observed 7-HQ fluorescence was minimal, a reduction of 1% was found at 1 mM CHAPS, which is far in excess of the actual amounts that would have been present in the assay. When the effect of 0.1% w/v E911, was investigated, it was found to reduce the observed 7-HQ fluorescence by 10%, though this concentration is again much higher than would have been present in the actual assay. Since the quenching of 7-HQ fluorescence by E911 is minimal, the fact that a smaller increase in fluorescence was observed with time would suggest that E911 is an inhibitor of P450 3A4, as has been reported by Hosea and Guengerich (Hosea and Guengerich, 1998).

The data generated in this assay can only be compared internally, because there is no published data of turnover numbers for this substrate with purified P450 3A4 in a non-reconstituted system. However, in order to get an idea of the upper limit of this rate, V_max values of 44 (no SD) and 25.2 +/- 6.5 have been reported by Miller et al.
(published at GENTEST web site www.gentest.com) and Lu et al (Lu et al, 2001), respectively, for fully reconstituted microsomal systems, i.e. P450 3A4 and CPR, in the case of Lu et al, 2001, and also cytochrome b₅, in the case of Miller et al. In comparison to these values, the rates of all the preparations seem very low, but it should be noted that cumene hydroperoxide has been reported not to yield very high rates, even when part of a fully reconstituted system (Yamazaki et al., 1995). Another potential explanation of these results is that substrate access to purified P450 3A4 is much slower than that to microsomal P450 3A4, which may be because the purified state is more aggregated than the microsomal one.

3.4 Conclusions

The samples of P450 3A4 generated by methods A1, A2 and B have been shown to be essentially pure by SDS-PAGE. The final yields for methods A1 and A2 were quite similar, cf. 95 to 80 nmol per litre of culture for method A1 and A2, respectively (Table 3-4). This is because when E911 was used losses were incurred on the metal affinity step, which did not happen in the presence of CHAPS; however, the reverse was true with the HiTrap Q column where increased fractionation of the CHAPS solubilised samples caused a reduction in the yield.

The fact that less P450 3A4 binds to the metal affinity column in the presence of E911, when compared to CHAPS, could be explained in terms of the protein’s aggregation state. If P450 3A4 were less aggregated when in the presence of 0.1% w/v E911, in comparison to 10 mM CHAPS, then there are fewer histidine tags present to bind to the metal affinity column. Therefore, if there are impurities present that can bind to a histidine tag, then these smaller P450 3A4 aggregates could be saturated by them and thus not interact at all with the affinity column. Alternatively, the aggregated CHAPS solubilised protein would have an improved chance of binding to the affinity column because it has a greater number of P450 3A4 monomers.

Method B was a better route than method A because it produced the highest yield (135 +/- 15 nmol/L) and reduced the chances of protein degradation, since the protein could be purified in one day instead of three. In addition, it highlighted the fact that
Table 3-4: Yields of P450 3A4 during purification.

Values represent mean +/- standard deviation, based on three experiments.

\( ^a \) this represents the total P450 3A4 recovered after this step for further characterisation.

P450 3A4 is aggregated under the conditions employed here, and that the size of the oligomer is dependent on the final CHAPS concentration used.

In addition, it has been found that both E911 and CHAPS produce an optical change, which is indicative of the fact that they are able to bind to the active site of P450 3A4. In the case of E911, there is strong evidence to support this since Triton N-101, a detergent with a similar structure, is a substrate for P450 3A4.

Finally, since all the samples of P450 3A4 contained minimal P420 and were able to oxidise the pro-fluorescent substrate 7-BQ to 7-HQ, it was assumed that they were active and thus could be used for the optical binding and NMR relaxation experiments.
CHAPTER FOUR

Substrate binding studies using optical and NMR spectroscopy
Substrate binding studies using optical and NMR spectroscopy

4.1 Introduction

This chapter will clarify why an alternative detergent and route of purification was adopted in an attempt to obtain an NMR active protein sample, *i.e.* one that yields changes in the spin lattice relaxation rate, $R_1$, for resonances of a bound substrate and that is in fast exchange with that compound. The results for this chapter will be divided up into two main sections: optical and NMR spectroscopy. The data obtained for the following samples of P450 3A4 will be presented and discussed:

i) E911, Q, flow through and 50 mM KCl fractions  
ii) CHAPS, Q, 50 mM and 100 mM KCl fractions  
iii) 2 mM CHAPS, S200

The sample of P450 3A4 (0.5 mM CHAPS, S200) will not be discussed in this chapter, as it was used purely for the optical binding experiments that are presented in Chapter Five.

The chapter will be concluded with a discussion of the NMR technique.

4.1.1 Investigating substrate binding using optical spectroscopy

The optical binding experiments were carried out for three main reasons:

1) To deduce whether or not the substrate under investigation could get close to the iron; if this were the case, a spin state shift should occur.
2) To determine a dissociation constant for this process, so that it could be compared to the value obtained by NMR.
3) To deduce the percentage high spin of the P450-substrate complex, so that an appropriate S value could be used in the NMR analysis.
4.1.2 Introduction to optical substrate binding

Schenkman et al (Schenkman et al, 1967) first classified the optical difference spectra observed upon substrate binding into three distinct types. A type I change is characterised by a minimum at 418nm and a maximum at 390nm, which is brought about by the displacement of water, i.e. a low to high spin state change (Figure 4-1). The majority of compounds that are metabolised by P450 give rise to this change on binding. Some compounds, e.g. alcohols, ketones and certain drugs e.g. phenacetin (Schenkman et al, 1972), produce the reverse of this i.e. a maximum at 420nm and a minimum at 385-390; this type is called reverse type I. This change is believed to occur because the substrate added stabilises the water to iron coordination bond in a mixed spin state system, i.e. this is a high to low spin state change. Finally, another group of compounds was found to shift the Soret band to longer wavelengths. These molecules, in the difference spectrum, give absorption maxima at 425-445nm and minima at 390-420nm. A change of this kind is called type II and is caused by direct interaction of the compound, usually through a nitrogen atom, with the haem (Schenkman et al, 1967; Ullrich et al, 1975). Many of these molecules are inhibitors, since they prevent oxygen from coordinating to the haem, which is a prerequisite for metabolism to occur. Each of these substrate-induced spectral changes (Figure 4-2) can be used to calculate an apparent dissociation constant (K_d) and an extrapolated maximum absorbance change at infinite substrate concentration (ΔA_{max}).

4.1.2.1 Determining K_d and ΔA_{max} values from optical data (Clarke, 1996)

By solving the quadratic solution of Equation 4-1, for the case where substrate concentration is represented as a function of the change in 390-418 absorbance (ΔA), Equation 4-2 can be derived. This equation was used initially to determine the K_d and ΔA_{max} values for each P450 3A4-substrate combination.
Figure 4-1: **UV Visible spectrum (300-500nm) of P450 101.**

- **a)** Low spin form. P450 101 diluted into 50 mM Tris pH 7.5. (Westlake, 1997, unpublished results).
- **b)** High spin form. P450 101 diluted into 50 mM Tris, pH 7.5, 1 mM camphor, 200 mM KCl, respectively (same as above).

Figure 4-2: **Difference spectrum changes observed upon substrate binding.**

The difference spectra were obtained as follows:
- **Type I** - Testosterone binding to P450 3A4 (2 mM CHAPS, S200).
- **Reverse type I** - Mathematical simulation using data for type I optical change.
- **Type II** - Isonazid binding to P450 3A4 (E911 purified) (Haldane, 2000, unpublished results).
where \([E_f], [S_f]\), and \([S_b]\) are the concentrations of free enzyme, free substrate and bound substrate, respectively. Note that \([E_f] = [E_T] - [S_b]\), where \([E_T]\) is total enzyme concentration and \([S_f] = [S_T] - [S_b]\), where \([S_T]\) is total substrate concentration.

\[
\Delta A = \frac{\Delta A_{\text{max}}}{2 \times [E_T]} \left[ (E_T + [S_T] + K_d) - 4 \times [E_T] \times [S_T] \right]^{1/2}
\]

\textit{Equation 4-2}

Equation 4-2 describes a rectangular hyperbola, and so will be called the hyperbolic equation.

In order to determine whether a substrate demonstrated homotropic cooperativity towards P450 3A4, the Hill equation, equation 4-3, was employed. This equation can be derived by assuming that the dissociation constant is much greater than the total enzyme concentration, i.e. the free substrate concentration is effectively equal to the total substrate concentration. Since all of the substrates in this study bind quite weakly, this approximation is a valid one.

\[
\Delta A = \frac{\Delta A_{\text{max}} \times [S_T]^n}{K_{0.5} + [S_T]^n}
\]

\textit{Equation 4-3}

where \(K_{0.5}\) is the substrate concentration at half saturation of the enzyme and \(n\) is the Hill coefficient.

Equation 4-3 provides an empirical fit to the data, and is useful for quantifying the degree to which a system is cooperative. The Hill coefficient or \(n\) value is the measure of this cooperativity, and for a system that is positively cooperative it has a value of greater than unity. The maximum number that \(n\) can reach is equal to the number of sites per protein molecule. The limitation of this equation is that, if the system is cooperative, it clearly cannot yield a dissociation constant for each separate binding event that is involved and
instead generates a $K_{0.5}$, which is purely a numerical value with no physical significance. As a result, although this value does give some indication as to how tightly the substrate is binding it can only be compared to other $K_{0.5}$ values for systems with similar $n$ values. Since equation 4-3 is used to investigate cooperativity, it will be referred to as the cooperative equation.

4.1.2.2 Determining the percentage high spin content for P450-substrate complexes from optical binding data

In order to calculate the distances of each proton from the haem the spin number (S) of the system needs to be known (see section 4.1.3.4, equation 4-5). Each unpaired electron has a spin number of 1/2, and so the total spin numbers for iron (III), which has five d-orbital electrons, in a low or high spin state are 1/2 or 5/2, respectively. The fraction of high spin present in a sample, and hence the effective spin number, can be estimated from $\Delta A_{\text{max}}$ if an extinction coefficient for the spin state change is known. However, in the case of P450 3A4 such a value has not been determined. Therefore, the high spin content was deduced by comparing the spectra of the various P450 3A4-substrate complexes with mathematical simulations of P450 101, based on their 390nm/418nm values. This is exactly the same method that was employed in chapter three to estimate the high spin content of the P450 3A4 samples after purification (see chapter three, section 3.3.1.2).

4.1.3 Investigating substrate binding using NMR spectroscopy

Since there is no crystal structure of P450 3A4 available the use of a series of indirect methods, such as kinetics, optical spectroscopy, site-directed mutagenesis and theoretical models, has been used to investigate this enzyme's active site structure (Shou et al, 1994; Harlow and Halpert, 1997; He et al, 1997; Szklarz and Halpert, 1997; Korzekwa et al, 1998; Harlow and Halpert, 1998; Hosea et al, 2000; Lu et al, 2001; Kenworthy et al., 2001). However, none of these techniques report on the substrate's position within the active site, which is crucial for producing an accurate model.

Proton NMR, on the other hand, has been used successfully to determine the positions of substrates relative to the active site iron with the following substrate-P450 pairs: laurate and 12-bromolaurate with P450 102 (Modi et al., 1995); codeine with P450 2D6 (Modi et al., 1996a); tienilic acid, lauric acid, and diclofenac with P450 2C9 (Poli-Scaife et al, 1997); and caffeine with P450 1A2 (Regal and Nelson, 2000). Thus, in the present study,
in an attempt to obtain distance constraints for a series of substrates so that a theoretical model of the active site structure of P450 3A4 could be developed to investigate substrate binding.

For the purposes of this investigation, it is important to understand the processes involved in proton relaxation, *i.e.* the spin-lattice relaxation time ($T_1$) and the spin-spin relaxation time ($T_2$), and how $T_1$ is determined, since this is the parameter that will be used to determine substrate proton distances to the iron.

### 4.1.3.1 Relaxation and correlation times

A single proton nucleus in the presence of a magnetic field $B_0$ may adopt one of two orientations, due to the quantisation of its angular momentum. Thus, the proton spin may be aligned with the magnetic field, the lower energy state, or against it, the higher energy state (Figure 4-3). However, the energy difference between these states is very small; thus, their populations at equilibrium, given by the Boltzmann distribution, are nearly equal.

In addition to this, the magnetic field $B_0$ causes the proton to precess about the direction of the field at a particular rate and angle. The rate at which a particular nucleus precesses about $B_0$ is called its Larmor frequency, and this is directly proportional to the field strength.

When many protons are in the presence of an applied magnetic field $B_0$ the majority will adopt the orientation of lowest energy, *i.e.* align with $B_0$, and so there will be a small net magnetization ($M_z$) along the axis of $B_0$, which is defined as the z axis (Figure 4-4).

When a radio frequency pulse $B_1$, which is equivalent to the Larmor frequency, is applied along the xy plane, the protons are given sufficient energy to move to an alternative orientation. Since there are more protons in the lower energy orientation, there will be a greater number of protons moving to the higher energy orientation, and thus $M_z$ will be reduced. In addition, the transitions, caused by $B_1$, bring the protons into phase with $B_1$ and hence with each other, so that they precess together. This concerted precession is
Figure 4-3: Vector diagram of the two allowed orientations of a proton nucleus in the presence of an applied magnetic field $B_0$.

Figure 4-4: Vector diagram of many protons precessing about a magnetic field $B_0$ resulting in net magnetisation along z-axis.

called phase coherence, and it results in increasing $M_{xy}$, *i.e.* net magnetisation in the xy plane at the Larmor frequency (Figure 4-5 i). The detection of $M_{xy}$ and hence of phase coherence, is the purpose of the NMR spectrometer. The addition of $M_z$ to $M_{xy}$ produces the resultant magnetisation $M$ (Figure 4-5 ii).
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i) Phase coherence caused by \( B_1 \) resulting in magnetisation in xy plane. The thin arrows represent precessing proton nuclei.

ii) Resultant magnetisation (M) produced by net magnetisation along both z and xy axes.

Figure 4-5: Magnetisation in xy plane caused by phase coherence.

---

\( B_0 \) is the magnetic field that is present in (a)-(c), and \( B_1 \) is the radio frequency pulse applied along the xy-plane.

Figure 4-6: Vector diagram illustrating the effect of a radio frequency pulse on the net magnetisation of a group of precessing protons over time.

---

If the radio frequency pulse is given for the right length of time there will be a moment at which there are equal numbers of protons in both orientations and the resultant magnetization will be entirely along the xy-plane; this is called a 90° pulse (Figure 4-6).
The length of time that the pulse is applied for determines the angle through which the resultant magnetisation has been tilted away from the $z$ axis.

Once the pulse $B_1$ is removed, however, the system will return or relax back to its equilibrium state over time, which could be from milliseconds to minutes depending on the system. This relaxation process is a combination of two mechanisms, both of which lead to an exponential decay.

1) $M_z$ increases exponentially to its original value. The time taken for this to occur is called the spin-lattice relaxation time, $T_1$.
2) $M_{xy}$ decays exponentially to zero. The time taken for this to occur is called the transverse relaxation time, $T_2$.

Relaxation only occurs due to many individual protons separately undergoing transitions between their allowed orientations. Taken together, these transitions restore the equilibrium of the system. However, the probability of these transitions occurring spontaneously is negligible; thus, the transitions that give rise to relaxation must be caused in some way. Two main sources of magnetic radiation have components at the Larmor frequency, and so could give rise to these transitions:

Firstly, there is general magnetic noise. Every molecule in the sample contains moving charges in the form of orbiting electrons and precessing nuclei and so could provide the energy required to bring about a transition. The frequency range of magnetic noise will depend upon the rate at which the molecules in solution move, which is affected by the molecular size and shape, temperature and the viscosity of the solution. The rate of molecular motion can be described quantitatively by translational and rotational correlation times, which are equivalent to the time taken for the molecule to translate through one molecular diameter or rotate through one radian. Generally, for a rigid spherical molecule, the correlation time increases linearly with molecular weight.

This random magnetic noise causes both $T_1$ and $T_2$ relaxation to occur. If the arrow in Figure 4-7 represents the Larmor frequency of a nucleus, then relaxation caused by magnetic noise is more rapid in a sample of intermediate rather than slow- or fast-moving
molecules. As a result, molecules with an intermediate correlation time will have the shortest $T_1$. They are also more efficient than fast moving molecules at causing $T_2$ relaxation and so, in the fast-to-intermediate region of molecular motion, both $T_1$ and $T_2$ are related in the same way to correlation time (Figure 4-8).
Random magnetic noise causes almost all $T_1$ relaxation to occur. However, $T_2$ relaxation has an additional contribution in the presence of slow-moving molecules, which is called spin-exchange and this is the second source of magnetic radiation. This process occurs most efficiently when nuclei spend a long time close to one another in the same mutual orientation with respect to $B_0$. Under these conditions, nuclei that have the same Larmor frequency can mutually exchange energy and thus will both undergo a transition. This process usually results in fewer nuclei precessing together, i.e. a loss in phase coherence, and so a reduction in $M_{xy}$. However, because the relative populations of the low and high energy states will remain unaltered there is no effect on $M_z$, and so therefore there is no contribution to $T_1$. Since this mechanism of relaxation becomes more efficient the longer the nuclei stay close together, $T_2$ continues to decrease with correlation time (Figure 4-8).

The relaxation time has a very significant impact on the observed signal. The energy of a state that is occupied for a time $t$ is uncertain by $\delta E$, i.e. the Heisenberg uncertainty principle, where the product of $\delta E$ and $t$ is greater than $h/2\pi$. Therefore, if the relaxation rate is very fast, i.e. $t$ is very small because the perturbed state is not occupied for long, $\delta E$ will be very large. When the energy of this transition is plotted on a graph, as in an NMR spectrum, it will appear as a broad band. Thus, the shorter the relaxation time, the broader the line will be.

4.1.3.2 Inversion recovery experiment (Determining $T_1$)

The NMR experiment for determining $T_1$, and thus $R_1$, values is made up of a 180° pulse, followed by a time delay, $\tau$, and finally another 90° pulse, i.e. 180°-$\tau$-90° or an inversion recovery experiment. The 180° pulse inverts the net magnetization onto the -z-axis, which then relaxes exponentially back to the +z-axis. By applying a 90° pulse after the time delay it is possible to monitor the progress of this form of relaxation. Thus, if a very short time delay is used ($\tau_0$) the 90° pulse will move the net magnetization onto the -xy-plane and the resultant signal will be -I. If, on the other hand, a very long delay is used ($\tau_x$) the nuclei will fully relax back to their equilibrium state and the 90° pulse will move the net magnetisation onto the +xy-plane and give a resultant signal of +I. When a series of such experiments are carried out, using a range of time delays from $\tau_0$ to $\tau_x$ the data describes the exponential increase of net magnetisation along the z-axis; thus, a value for $T_1$ can be determined for each resonance by plotting the natural logarithm of peak height against $\tau$ (Equation 4-4).
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180° pulse $\rightarrow$ time $\tau$ $\rightarrow$ partially relaxed $M_z$ $\rightarrow$ 90° pulse $\rightarrow$ Data acquisition

Part of the spectrum of valinomycin, showing partial relaxation with values of $\tau$ from 10 milliseconds (top) to 5000 milliseconds (bottom).

Figure 4-9: $T_1$ inversion recovery experiment.

The time delays $\tau_0$ and $\tau_s$ from the text are marked on.
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\[ I(t) = I(0) + P \cdot \exp(\tau \cdot \frac{1}{T_1}) \]

Equation 4-4

A rough \( T_1 \) can be found by estimating the \( \tau \) at which the signal intensity is zero, since \( \tau = 0.69T_1 \) at this point (Figure 4-9).

4.13.3 Exchange regime

The following explanation of how the exchange regime of a system can affect the data obtained by NMR will illustrate why it is so important that, in the present study, the substrate is in a very fast exchange regime, on the NMR time scale, with the protein.

The dynamic equilibrium between a substrate free in solution and a substrate bound to a P450 results in the molecule with its attached protons exchanging at some rate between two environments \( i.e. \) the bulk solution and the paramagnetic active site centre. The effect that this has on the spectrum depends on two factors:

1) The differences in NMR parameters (\( e.g. \) chemical shift, relaxation rate) between the two environments
2) The rate of exchange between them.

In the case of chemical shift, where exchange is very slow (compared to the difference in chemical shift between the two environments, expressed in Hz), a separate signal is observed in the NMR spectrum for each environment. When the exchange is very fast only a single peak is observed, and its position is a measure of the relative time spent in each environment; it is only in this regime that it is possible to obtain distance data from the \( T_1 \) measurements. If the system is in an intermediate exchange rate, the spectrum will vary between these two extremes. Figure 4-10(a)-(c) illustrate the three exchange regimes.

When considering the effect of exchange regime on \( T_1 \) relaxation times it is easier to use relaxation rates, \( R_i = 1/T_i \), since these are directly comparable. Thus, in the case where exchange is very slow (compared to the difference in relaxation rate between the two
(a), (b) and (c) depict fast, intermediate and slow exchange, respectively.

**Figure 4-10: Diagram of the effects of exchange rate on the NMR spectrum.**

environments, expressed in Hz) the protons from the two different environments, *i.e.* in bulk solution and bound to the protein, do not interact with each other and so two separate signals are observed. However, when a proton is very close to a paramagnetic centre, as is the case in this study, its relaxation time is dramatically shortened and therefore the resultant peak from this environment will be broadened to almost nothing. Thus, only the signal from the bulk solution will be observed and this yields no information about the distance that this proton is away from the iron. When the exchange is very fast, however, the protons from the two different environments can interact with each other and thus a single signal is observed, whose relaxation rate represents the weighted average of the proton in the free and bound state. If, however, the system is in intermediate exchange then only a fraction of the nuclei will interact with each other, and this fraction cannot be determined unless the $R_{1,b}$ value is already known.
Thus, the present NMR studies will only work when the system is in fast exchange with regard to both chemical and relaxation time scales, since it is only under these conditions that an \( R_{1,M} \) value can be obtained, which can then be used to determine a distance for that particular proton.

### 4.1.3.4 Paramagnetic effects on NMR

Paramagnetic effects on NMR spectra arise because an unpaired electron has a magnetic dipole moment that is 1836 times larger than that of a proton. As a result, dramatic effects in chemical shift (20-40ppm) and/or line broadening (10-100 fold) have been observed for nuclei that are close to a paramagnetic species (Jardetzky and Roberts, 1981). The interactions that can occur between a nucleus and an unpaired electron are dependent on where they are in relationship to one another:

a) Nucleus interacting with an unpaired electron that is on another nucleus. This is a through space interaction and the effect is directional. If this interaction does not change with time, it will produce a chemical shift, which is known as a pseudocontact shift. When the interaction is time-dependent, however, it will produce line-broadening effects through dipolar relaxation.

b) A single nucleus interacting with its own unpaired electron. This interaction exists only to the extent to which an unpaired electron dwells at a given nucleus. This is not directional and if it has a time-invariant component a chemical shift is produced, which is called a contact shift. When the interaction is time-dependent the fluctuations will produce scalar relaxation.

In the case of a substrate binding to a P450, it is the time-dependent through space interactions, *i.e.* type (a), which are being investigated. The line broadening effects caused by dipolar relaxation equates to reductions in the relaxation time, \( T_1 \) and \( T_2 \), or the inverse, an increase in the relaxation rate, \( R_1 \) or \( R_2 \). By measuring \( R_{1,\text{obs}} \), as a function of substrate concentration, it is possible to determine an \( R_{1,b} \) value using Equation 4-5:

\[
R_{1,\text{obs}} = \frac{[E_0]}{K_d + [S_0]} \left( R_{1,b} - R_{1,f} \right) + R_{1,f}
\]

*Equation 4-5*
where \([E_0]\) is the total enzyme concentration and \(K_d\) is the binding constant.

The \(R_{1,\text{obs}}\) value is equivalent to the \(R_{1,M}\) value, which is the relaxation rate of the proton when it is close to the paramagnetic centre, only when the off rate is much greater than \(R_{1,M}\), \(i.e.\) when the system is in fast exchange. From the \(R_{1,M}\) value the distance of a proton from the paramagnetic centre can be obtained by using the Solomon-Bloembergen equation, Equation 4-6.

\[
R_{1,M} = \frac{2}{15} \left( \frac{\mu_0}{4\pi} \right)^2 \frac{\gamma_1^2 g^2 S(S+1)\beta^2}{r^6} \left( \frac{3\tau_e}{1+\omega_1^2\tau_e^2} + \frac{7\tau_e}{1+\omega_0^2\tau_e^2} \right)
\]

*Equation 4-6*

where \(\mu_0\) is the vacuum permeability constant, \(\gamma_1\) is the nuclear gyromagnetic ratio, \(g\) is a constant that describes the electronic environment, \(S\) is the total electron spin, \(\beta\) is the Bohr magneton, \(\omega_1\) and \(\omega_S\) are the nuclear and electronic Larmor frequencies, respectively, \(\tau_e\) and \(\tau_c\) are correlation times that describe the fluctuations of the dipolar and scalar interactions, respectively, and \(r\) is the distance between the nucleus and the electron.

This equation assumes, however, that the system only contains one unpaired electron and that it is in a highly symmetrical environment, since when there are two or more unpaired electrons, \(e.g.\) in the high spin state there would be five, these might interact in an anisotropic way, and so this would have to be accounted for in the equation as well. Thus, it would be more accurate to apply equation 4-4 to a P450 in the low spin state with a substrate bound, \(i.e.\) use a substrate that does not affect the haem so as to bring about a change from low to high spin. Interestingly, there are some substrates that bind without altering the spin state of P450s (Pirrwitz et al., 1982) and such interactions have also been observed in the present work, see chapter five. Also, the equation assumes a point location for the unpaired electrons, and so if these electrons were delocalised across the haem it would no longer be valid.

Despite these possible flaws, this equation has been used to determine haem iron-substrate proton distances for various P450s (Modi et al., 1995), (Modi et al., 1996a), (Poli-Scaife et al, 1997), (Regal and Nelson, 2000). Indeed, the reliability of this equation has been demonstrated by the fact that both NMR (Modi et al., 1995) and crystallography (Li and
that the proton, at which a fatty acid is metabolised, binds at a distance of 7.5-7.9Å away from the haem iron of oxidised P450 102.

4.1.3.5 Evaluating the suitability of a substrate-paramagnetic centre system for analysis by NMR spectroscopy

In order to establish whether paramagnetic centre to substrate proton distances can be determined for a particular system, e.g. P450 3A4-testosterone, the following procedure should be carried out:

Firstly, the substrate's proton NMR spectrum should be obtained and assigned. In order for the substrate to be suitable for analysis by NMR it must have well resolved resonances. Since, if two or more resonances overlap the relaxation data obtained would not be a single exponential decay, instead it would be a complicated combination of exponentials that might not be easily deconvoluted, and thus the resultant $R_{1,obs}$ value would not be very accurate. Secondly, the exchange regime of the system should be determined by obtaining $R_{1,b}$ at two different temperatures. If $R_{1,b}$ decreases with increasing temperature it is determined by $R_{1,M}$ and the system is in fast exchange, whereas if $R_{1,b}$ increases with increasing temperature it is determined by the off rate and the system is in slow exchange.

4.1.4 Substrates used in study

4.1.4.1 7-benzyloxyquinoline

Although 7-benzyloxyquinoline (7-BQ) was not used for the NMR experiments it was used in the optical binding experiments to demonstrate that substrates could access the active site. The optical binding data obtained for 7-BQ have been used to illustrate how the results from the hyperbolic and cooperative equations were interpreted to evaluate the type of binding interaction; this same method was then applied for all the other substrates. In addition, the 7-BQ data set has been used to demonstrate how the percentage high spin data were obtained for each substrate.

4.1.4.2 Testosterone

Testosterone was considered to be the best substrate to investigate first, since it had been used successfully in the past in this laboratory to obtain distances between the haem iron
and substrate protons using NMR (Modi, 1996, unpublished results). Also this substrate demonstrates both homotropic and heterotropic cooperativity with P450 3A4 (He et al., 1997; Harlow and Halpert, 1997; Ueng et al., 1997; Domanski et al., 1998; Harlow and Halpert, 1998; Domanski et al., 2000; Hosea et al., 2000; Wang et al., 2000; Lu et al., 2001; Kenworthy et al., 2001; Domanski et al., 2001), and so this compound could be used to investigate these properties of P450 3A4 by NMR.

4.1.4.3 2-hydroxypropyl β-cyclodextrin encapsulated testosterone

Testosterone encapsulated in 2-hydroxypropyl β-cyclodextrin, which will be referred to as encapsulated testosterone, is commercially available from Sigma-Aldrich. Cyclodextrins are cyclic oligosaccharides that possess an internal cavity that is hydrophobic but which also have an external surface that is hydrophilic (Figure 4-11). In Figure 4-11 i) the empirical structure of cyclodextrins are shown. The repeating unit is a D-glucopyranoside molecule, and the number of these units present in a cyclodextrin determines its name (Steed and Atwood, 2000)

In Figure 4-11 ii) a three dimensional structure of β-cyclodextrin is shown in order to illustrate the cavity that is generated, which can be filled by a suitably sized hydrophobic molecule (Albers and Muller, 1992; Redenti et al, 2000; Loftsson et al, 2002; Perdomo-Lopez et al, 2002) such as testosterone in this particular instance.

4.1.4.4 Amitriptyline

In order to avoid the necessity for any additional solubilising agent amitriptyline was investigated as a suitable substrate (Ueng et al., 1997) for the NMR experiments, since the hydrochloride salt is quite soluble in water (Budavari, 1996).

4.2 Results and discussion of optical substrate binding

This section will be subdivided into the results obtained for each substrate: 1) 7-benzyloxyquinoline, 2) testosterone, 3) testosterone encapsulated in 2-hydroxypropyl β-cyclodextrin, and 4) amitriptyline. For each substrate/P450 combination, a $K_d$, $\Delta A_{\text{max}}$, $n$ value, and percentage high spin was determined, unless stated otherwise.
4.2.1 7-BQ; Determining $K_d$ and $\Delta A_{\text{max}}$ using the hyperbolic equation

The substrate was found not to absorb between 450nm and 390nm, within the concentration range of interest (0-430 µM), and thus it was not necessary to correct for this absorption by adding the same concentration to the reference cuvette. However, below 390nm, the substrate did absorb slightly and this gave rise to the increase in absorbance between 360nm and 350nm observed in the difference spectra (Figure 4-12).

The addition of 7-BQ to P450 3A4 (all methods) produced a type I binding spectrum (Figure 4-12). When the hyperbolic equation was fitted to these data (Figure 4-13 i-iii and Table 4-1), it was found that a $K_d$ could not be determined for the samples of protein prepared using E911, because the protein did not reach a point at which it was saturated by the substrate (Figure 4-13 i). In addition, it could be seen that the hyperbolic equation
provided a poor fit to the data obtained using P450 3A4 (CHAPS, Q, 50 mM KCl)) and P450 3A4 (2 mM CHAPS, S200) (Figure 4-13 ii and iii), since the standard deviation on the Kd obtained for these samples was quite large, approximately 21% for the Q, 50 mM KCl sample and 15% for the S200 sample.

4.2.1.1 Determining $K_d$, $\Delta A_{\text{max}}$ and n using the cooperative equation

The plots of the binding data for P450 3A4 (CHAPS, Q, 50 mM KCl) and P450 3A4 (2 mM CHAPS, S200) appeared to be more sigmoidal than hyperbolic, especially in the case of the latter (Figure 4-13 iii), and this suggested that the interaction may be cooperative. As a result, the cooperative equation was used to obtain a $K_d$, $\Delta A_{\text{max}}$ and an n value, i.e. the degree of cooperativity (Figure 4-14 and Table 4-2).

The cooperative equation yielded a far better fit than the hyperbolic equation, as could be seen by the fact that the standard deviation obtained for the P450 3A4 (CHAPS, Q, 50 mM KCl) and P450 3A4 (2 mM CHAPS, S200) samples was only 5% (cf. 21%) and 2% (cf. 15%), respectively. The values in brackets are the percentage standard deviation errors obtained when the hyperbolic equation was used. In addition, the $r^2$ values, which indicate the goodness of a fit, had increased in comparison to those obtained using the hyperbolic equation. The fact that the n values were greater than one supported the theory that the binding was cooperative. In addition, all of these samples of P450 3A4 show roughly the same degree of cooperativity, cf. 1.7 +/- 0.1 to 1.5 +/- 0.1, thus indicating that this property of the interaction between 7-BQ and P450 3A4 is not sensitive to the manner in which the protein is prepared.

Irrespective of the interaction being cooperative, the $K_{0.5}$ data suggested that 7-BQ binds equally tightly to both fractions of P450 3A4 (CHAPS, Q), cf. 192 μM and 203 μM, but more tightly to P450 3A4 (2 mM CHAPS, S200), which had a $K_{0.5}$ of 129 μM. However, it was also noted that the $\Delta A_{\text{max}}$ values for samples of P450 3A4 (CHAPS, Q) and P450 3A4 (2 mM CHAPS, S200) were not the same, cf. 0.118 or 0.125 and 0.079. Since this titration has been carried out under identical conditions and with approximately the same P450 3A4 concentration, the difference in $\Delta A_{\text{max}}$ could only be due to either inhomogeneous protein samples or an error within the fitting of the data. Since P450 3A4 elutes as separate bands from the HiTrap Q column and as a mixture of oligomers from the
Figure 4-12: Optical difference spectra obtained with 7-BQ.

Difference spectra for the following P450 3A4 samples:

i) E911, Q, flow through

ii) CHAPS, Q, 50 mM KCl

iii) 2 mM CHAPS, S200

P450 3A4 (1 μM) in KPB (0.1M), glycerol (5% w/v), methanol (3% w/v) pH 8, was titrated with 7-BQ using method 3. The data was zeroed at 450nm for presentation purposes. The black arrows mark the absorbance changes upon 7-BQ addition, and the red arrow indicates where 7-BQ absorbs.
Figure 4-13: Hyperbolic fits to the data obtained with 7-BQ.

Plots of absorbance change (390-418) against 7-BQ concentration for the following P450 3A4 samples:

i) E911, Q, flow through (●)

ii) CHAPS, Q, 50 mM KCl (○)

iii) 2mM CHAPS, S200 (▲)

The plotted data are from one optical titration experiment. The hyperbolic fit is the red line.

135
S200 column, these samples are clearly not homogeneous. Thus, if there were a component of these samples that bound 7-BQ less tightly, and there was a greater proportion of this component in the samples of P450 3A4 purified using the HiTrap Q column, then these samples would have a higher $K_d$ and $A_{\text{max}}$. Alternatively, the difference in $A_{\text{max}}$ could be due to an error in the extrapolation to this value using the cooperative equation. It can be seen from the fit of the data (Figure 4-14) that saturation of the P450 3A4 samples with 7-BQ was not reached. Since the P450 3A4 (CHAPS, HiTrap Q, 50 mM KCl) data set has fewer data points at higher concentration than the P450 (2 mM CHAPS, S200) data set, the equation could have overestimated the $A_{\text{max}}$ value. If it were assumed that the CHAPS protein samples actually have roughly the same shape of binding curve, then the $A_{\text{max}}$ would determine the $K_{0.5}$ value. By scaling the $A_{\text{max}}$ and $K_{0.5}$ of P450 3A4 (CHAPS, HiTrap Q, 50 mM KCl) so that the $A_{\text{max}}$ was the same as that of P450 3A4 (2 mM CHAPS, S200), the $K_{0.5}$ of P450 3A4 (CHAPS, HiTrap Q, 50 mM KCl) became 129 $\mu$M, thus the same as the P450 3A4 (2 mM CHAPS, S200) sample.

### Table 4-1: Dissociation constants determined for 7-BQ using the hyperbolic equation.

*a* it is impossible to estimate a value for this data set.

*n/a = non applicable*

The values obtained are for one data set, thus the standard deviations have been obtained from the fitting program.

<table>
<thead>
<tr>
<th>Sample of P450 3A4</th>
<th>$K_d / \mu$M</th>
<th>$A_{\text{max}}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>E911, Q, flow through</td>
<td>a</td>
<td>a</td>
<td>n/a</td>
</tr>
<tr>
<td>E911, Q, 50 mM KCl</td>
<td>a</td>
<td>a</td>
<td>n/a</td>
</tr>
<tr>
<td>CHAPS, Q, 50 mM KCl</td>
<td>595 +/- 125  (21%)</td>
<td>0.226 +/- 0.031</td>
<td>0.993</td>
</tr>
<tr>
<td>CHAPS, Q, 100 mM KCl</td>
<td>625 +/- 145  (23%)</td>
<td>0.238 +/- 0.039</td>
<td>0.992</td>
</tr>
<tr>
<td>2 mM CHAPS, S200</td>
<td>302 +/- 46   (15%)</td>
<td>0.121 +/- 0.001</td>
<td>0.987</td>
</tr>
</tbody>
</table>
Figure 4-14: Comparing the fits obtained with the cooperative and hyperbolic equations for the 7-BQ data set.

Plots of absorbance change (390-418) against 7-BQ concentration for the following P450 3A4 samples:

i) CHAPS, Q, 50 mM KCl (○)

ii) 2mM CHAPS, S200 (▲)

The plotted data are from one optical titration experiment.
Table 4-2: Dissociation constants determined for 7-BQ using the cooperative equation.

K_{0.5} is a numerical value for the concentration of substrate at half ΔA_{max} rather than a dissociation constant.

The values obtained are for one data set, thus the standard deviations have been obtained from the fitting program.

<table>
<thead>
<tr>
<th>P450 3A4 sample</th>
<th>K_{0.5}/μM (^a)</th>
<th>A_{max}</th>
<th>n</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPS, Q, 50 mM KCl</td>
<td>192 +/- 10 (5%)</td>
<td>0.118 +/- 0.004</td>
<td>1.5 +/- 0.1</td>
<td>0.999</td>
</tr>
<tr>
<td>CHAPS, Q, 100 mM KCl</td>
<td>203 +/- 14 (7%)</td>
<td>0.125 +/- 0.006</td>
<td>1.5 +/- 0.1</td>
<td>0.999</td>
</tr>
<tr>
<td>2 mM CHAPS, S200</td>
<td>129 +/- 3 (2%)</td>
<td>0.079 +/- 0.001</td>
<td>1.7 +/- 0.1</td>
<td>0.999</td>
</tr>
</tbody>
</table>

No other groups have performed optical binding experiments upon P450 3A4 and 7-BQ and so there are no K_d data available for comparison.

4.2.1.2 Determining the spin state of the P450 3A4-7-BQ complex

Spectral comparisons between the P450 3A4-7-BQ complexes and the simulations of P450 101 were made (Figure 4-15), based on the closest 390nm/418nm absorbance value, and the percentage high spin P450 was determined for each sample (Table 4-3). Since similar 390/418 values of P450 3A4-7-BQ and P450 101 gave approximately the same UV/Vis spectra, and therefore could be used to determine a good estimate of the percentage high spin present in the sample, this comparison method was utilised for all the substrates investigated.

4.2.2 Testosterone; Determining K_d, ΔA_{max} and n

Testosterone appeared to be soluble up to about 0.4 mM in 0.1 M KPB, 5% v/v glycerol, 10% v/v DMSO pH 8, which was the buffer system used previously for the NMR experiments. The organic solvent DMSO was used in order to assist the dissolution of testosterone into the aqueous medium, since it is a highly hydrophobic compound that is
only soluble in water at concentrations below 130 µM (Dawson et al., 1986), which would be too low for the NMR experiments. Other research groups have reported using concentrations of testosterone up to 1 mM (Ueng et al., 1997; Wang et al., 1997), however, these are microsomal systems where it is likely that testosterone can partition into the membranes and thus more can be added to the aqueous medium. In addition, microsomal samples are and non-transparent, unlike the dilute samples of purified P450 3A4 employed for optical or NMR experiments, thus making it very difficult to observe precipitation. This buffer system was only used, however, for P450 3A4 (E911, Q, both fractions) in the optical binding (Figure 4-16i) because it was found that it did not yield the expected NMR results. It has been reported that 1% v/v DMSO can weakly affect (<10% inhibition) the $K_m$ of the reaction between liver microsomal P450 3A4 and dextromethorphan (Hickman et al., 1998), and that 3% v/v DMSO can inhibit the activity of microsomal recombinant P450 3A4 towards testosterone by 67% (Busby et al., 1999), therefore the DMSO concentration was reduced. On further investigation, it was found that 5% v/v DMSO was still capable of dissolving testosterone to 0.4 mM and resulted in larger type I optical changes upon binding with P450 3A4 (E911, Q, both fractions) (Figure 4-16ii). It was also noted that testosterone did not absorb in the 350-450nm region, thus there was no need to add this substrate to the reference cuvette.

The same buffer system containing 5% v/v DMSO was then used with the other samples of protein, i.e. P450 3A4 (CHAPS, Q, both fractions) and P450 3A4 (2 mM CHAPS, S200). These samples also gave rise to type I optical spin state changes upon binding; although

<table>
<thead>
<tr>
<th>P450 3A4 sample</th>
<th>390nm/418nm</th>
<th>% High Spin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3A4</td>
<td>101</td>
</tr>
<tr>
<td>E911, Q, both fractions</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>CHAPS, Q, both fractions</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2 mM CHAPS, S200</td>
<td>1.04</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Table 4-3: Spin state data for the P450 3A4-7-BQ complexes.
the magnitude of the changes were larger than in the case of P450 3A4 (E911, Q, flow through) (Figure 4-17). The optical binding data for all the protein samples were analysed using the hyperbolic and cooperative (Figure 4-17) and the results compared (Table 4-4). It was found that the E911-purified protein was not saturated by testosterone and so it was concluded that the dissociation constant was at least 160 μM. In contrast, the CHAPS-purified samples were able to bind testosterone more tightly and so were almost saturated by it within the same concentration range (0-325 μM).

Figure 4-16: Optical binding data for P450 3A4 (E911, Q, flow through) with testosterone.

i) Difference spectra. Data zeroed at 450nm for presentation purposes.

ii) Plots of absorbance change (390-418) against testosterone concentration.

1 μM P450 3A4 in 0.1MKPB, 5% v/v glycerol, (●) 10 or (○) 5% v/v DMSO, pH 8, was titrated with testosterone using method 3. The plotted data are from one optical titration experiment.
Figure 4-17: Plot of $\Delta(390-418)\text{Absorbance}$ vs. concentration of testosterone for all protein samples.

Plots of absorbance change (390-418) against testosterone (TES) concentration for the following P450 3A4 samples:

i) E911, Q, flow through (•)

ii) CHAPS, Q, 50 mM KCl (○)

iii) 2 mM CHAPS, S200 (▲)

P450 3A4 (1 μM) in KP B (0.1M), glycerol (5% v/v), DMSO (5% v/v) pH 8, was titrated with testosterone using method 3. The plotted data are from one optical titration experiment. The hyperbolic and coop NL fits are the red and blue lines, respectively.
Table 4-4: Optical binding data determined for testosterone.

The values obtained are for one data set, thus the standard deviations have been obtained from the fitting program.

By using the hyperbolic equation, it was found that both samples of P450 3A4 (CHAPS, Q) bound testosterone equally tightly, cf. 100 +/- 2 (2%) and 106 +/- 3 μM (3%) for the 50 and 100 mM KCl fractions, respectively. The low percentage errors on the standard deviations suggested that this was an appropriate equation to fit the data to and that the interaction was not cooperative. This result was verified by fitting the cooperative equation to the data, since the n values obtained were not significantly different from one, and the K0.5s were approximately the same as those obtained for the hyperbolic equation, cf. 100 +/- 2 (2%) and 96 +/- 4 (4%) for P450 3A4 (CHAPS, Q, 50 mM KCl).

For P450 3A4 (2 mM CHAPS, S200), a dissociation constant of 147 +/- 9 (6%) μM was determined by applying the hyperbolic equation, which indicated that this sample bound
<table>
<thead>
<tr>
<th>Reference:</th>
<th>$K_0 / \mu M$</th>
<th>$\Delta A_{\text{max}(390-418)}/\mu M$ P450 3A4</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Harlow and Halpert, 1998)$^a$</td>
<td>56 +/- 3</td>
<td>0.08 +/- 0.001</td>
<td>n/a</td>
</tr>
<tr>
<td>(Hosea et al., 2000)$^b$</td>
<td>41 +/- 11</td>
<td>NR</td>
<td>1.3 +/- 0.1</td>
</tr>
</tbody>
</table>

**Table 4-5: Reported optical binding data for testosterone.**

$^a$ This experiment used 0.5 $\mu M$ CHAPS purified P450 3A4 and was carried out in 50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (pH 7.6), 0.1 mg/mL dioleoyl phosphatidylcholine, and 0.05 % CHAPS. A 1/100th volume of various dilutions of testosterone in methanol (or methanol alone) was added to nine separate aliquots of P450 3A4.

$^b$ This experiment used 1 $\mu M$ CHAPS purified P450 3A4 and was carried out in 0.1 M KPB pH 7.4. Testosterone, dissolved, in methanol, was added to the P450 3A4 solution up to a concentration of 200 $\mu M$. The final methanol concentration was $\leq 2\%$ v/v.

All these data were derived using the cooperative equation.

NR = Not reported.

testosterone less tightly than both the samples of P450 3A4 (CHAPS, Q). However, the larger percentage error on this $K_0$ in comparison to the other samples, $cf$. 6% for this sample with 2% for P450 3A4 (CHAPS, Q, 50 mM KCl), and the slightly lower $r^2$ value of 0.997, in comparison to 0.999 for both other samples, suggested that the equation was not fitting the data as well. When the cooperative equation was applied the $K_{0.5}$ decreased to 98 +/- 2 $\mu M$ (2%), the percentage error on the standard deviation was reduced and the $r^2$ value increased from 0.997 to 0.999, thus indicating that this is a more appropriate equation to fit the data to and that the interaction between testosterone and this sample of P450 3A4 is cooperative. It is worth noting that the $n$ value of 1.3 +/- 0.02 obtained here is identical to that reported by Hosea *et al* (Hosea et al., 2000)(Table 4-5), who also use the cooperative equation for their data analysis. Interestingly the $K_{0.5}$ and the $\Delta A_{\text{max}}$ value were now very similar to the values obtained for both samples of P450 3A4 (CHAPS, Q), $cf$. $K_{0.5}$ of 98 +/- 2 and $\Delta A_{\text{max}}$ of 0.098 +/- 0.001 for this sample with $K_0$ of 100 +/- 2 and
Chapter Four

<table>
<thead>
<tr>
<th>P450 3A4 sample</th>
<th>390/418</th>
<th>% High Spin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3A4</td>
<td>101</td>
</tr>
<tr>
<td>E911, Q, both fractions</td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>CHAPS, Q, both fractions</td>
<td>1.31</td>
<td>1.32</td>
</tr>
<tr>
<td>2 mM CHAPS, S200</td>
<td>1.25</td>
<td>1.26</td>
</tr>
</tbody>
</table>

Table 4-6: Spin state data for the P450 3A4-testosterone complexes.

The ΔA\text{max} of 0.102 +/- 0.001 for P450 3A4 (CHAPS, Q, 50 mM KCl). Therefore, the strength of the binding interaction between testosterone and the CHAPS-purified samples of P450 3A4 seems to be almost identical, but the type of interaction appears to be different.

By comparing the results to the literature (Table 4-5), it was found that the K\text{d} reported here is slightly higher than those found by other groups, but this could be because methanol was used instead of DMSO, and/or because it was used at a lower concentration, cf. 5% v/v DMSO (707 mM) and 2% v/v methanol (494 mM). However, the optical K\text{d} determined for the batch of P450 3A4 used previously in this laboratory for the NMR experiments (Modi, 1996, unpublished results) was 129 +/- 7 μM, which suggested that the P450 3A4 samples purified in CHAPS were more similar to this batch than the E911-purified samples. It was also found that the ΔA\text{max}/μM P450 3A4 values determined in this study were higher than the published values, cf. 0.098 for P450 3A4 (2 mM CHAPS, S200) with 0.08 (Harlow and Halpert, 1998). This could be due to errors in fitting the data, differences between inhomogeneous protein batches, and/or because the actual protein concentration used in these studies was greater than that employed by Harlow and Halpert (Harlow and Halpert, 1998), which could be because of inaccuracies in the P450 determination.

4.2.2.1 Determining percentage high spin

The 390/418 value for each P450 3A4-testosterone complex was determined and used to produce a simulation of P450 101 with a similar value, from this simulation a percentage
Figure 4-18: UV/Vis spectral analysis of P450 3A4-testosterone complexes.

a) P450 3A4 (E911, Q, flow through) with 0.322 mM testosterone.

b) Simulation of 55% high spin P450 101.

c) P450 3A4 (CHAPS, Q, 50mM KCl) with 0.329 mM testosterone.

d) Simulation of 72% high spin P450 101.

e) P450 3A4 (CHAPS, S200) with 0.327 mM testosterone

f) Simulation of 70% high spin P450 101.
high spin of the complex could be deduced (Table 4-6). Since the P450 101 simulations were visually similar to the P450 3A4-testosterone complexes, the percentage high spin deduced for each complex was considered to be a good estimate (Figure 4-18).

4.2.3 2-hydroxypropyl β–cyclodextrin encapsulated testosterone; Determining $K_d$, $\Delta \lambda_{\text{max}}$ n

In the case of P450 3A4 (E911, Q, both fractions), a cyclodextrin-encapsulated form of testosterone, which appeared to be soluble in buffer to at least 20 mM, was tried in order to eliminate the effects of the DMSO, since this was one of the possible reasons that testosterone was not saturating these samples of enzyme.

It is worth noting at the outset that any $K_d$ determined here would only be an apparent value, since testosterone is in a dynamic equilibrium with both the cyclodextrin and the protein.

Upon addition of up to 385 $\mu$M encapsulated testosterone to P450 3A4 (E911, Q, both fractions) a type I optical change was observed (Figure 4-19 i), but at higher concentrations both the 418nm and 390nm absorbances dropped and a small blue shift of about one nanometre occurred (Figure 4-19 ii). Due to this drop of absorbance at higher concentrations, it was not possible to use the hyperbolic equation to fit the data (Figure 4-20). It was thought that the changes that occurred at above 385 $\mu$M of encapsulated testosterone could be due to the cyclodextrin interacting with the protein in some way, and so a similar titration was carried out with 2-hydroxypropyl β–cyclodextrin (Figure 4-21). From this titration, it was observed that from 0-0.01% w/v cyclodextrin, which is equivalent to 0-385 $\mu$M encapsulated testosterone, the only change to occur was a decrease in the absorbance at 418nm, but above this concentration both 418nm and 390nm absorbances dropped and the whole Soret band was red shifted by approximately two nanometres (Figure 4-21 i). Due to this shift in the Soret band, the difference spectra produce a peak at 430nm (Figure 4-21 ii), but this is not a type II interaction since the peak in the absolute spectrum is at 420nm and not 424nm. These small changes in the Soret band suggest that 2-hydroxypropyl β–cyclodextrin does interact with P450 3A4 in some
Figure 4-19: Optical binding data for P450 3A4 (E911, Q, flow through) with encapsulated testosterone.

Optical difference spectra observed with P450 3A4 (E911, Q, flow through) upon addition of the following concentrations of encapsulated testosterone:

i) 0 to 385 μM

ii) 477 to 1750 μM.

1μM P450 3A4 in 0.1M KPB, 5%v/v glycerol, pH 8 at 27°C, was titrated with encapsulated testosterone using method 1. Data zeroed at 450nm for presentation purposes. The solid arrows mark the absorbance changes upon substrate addition.
Figure 4-20: Plot of Δ(390-418)Absorbance vs. concentration of encapsulated testosterone (TES) for P450 3A4 (E911, Q, flow through).

The plotted data are from one optical titration experiment.

way. There is no data to indicate whether this cyclodextrin is metabolized by P450 3A4, but since cyclosporin A, which is a molecule of equivalent size, can be oxidised by this P450 (Kronbach et al., 1988) the possibility does exist. However, since encapsulated testosterone produces a type I optical change, and at higher concentrations almost saturates P450 3A4 (E911, Q, flow through), if the cyclodextrin binds it does so less tightly than testosterone. One possible explanation of these results is that the whole cyclodextrin-testosterone complex enters the active site of P450 3A4, but only up to a certain point, and then testosterone leaves the cyclodextrin to occupy a site closer to the iron and cause a type I spin state change. When the effects of the cyclodextrin on the 390nm and 418nm absorbances were taken into account, the hyperbolic equation still did not fit the whole data set properly, which might suggest that the presence of both testosterone and the cyclodextrin have a synergistic effect (Figure 4-22 i). However, when the data was truncated at 830 µM encapsulated testosterone, it was possible to determine an estimate of the apparent Kd, which was 480 +/- 58 µM, r² = 0.992 (Figure 4-22 ii).

When this optical binding data was compared to that obtained using 5% v/v DMSO as the solubilising agent it could be seen that very similar absorbance changes occurred up to approximately 320 µM (Figure 4-23), which suggested that the DMSO was not the reason that testosterone could not saturate this sample of P450 3A4, further evidence for this was the fact that the CHAPS solubilised samples of this enzyme did.
Figure 4-21: Optical binding data for P450 3A4 (E911, Q, flow through) with 2-hydroxypropyl β-cyclodextrin.

i) Absolute spectra of P450 3A4 (E911, Q, flow through) in the absence of substrate (—), and in the presence of 0.01 (—) and 0.04 (—) %w/v 2-hydroxypropyl β-cyclodextrin. The values in the brackets are the equivalent encapsulated testosterone concentrations in micromolar.

ii) Difference spectra of P450 3A4 (E911, Q, flow through) upon addition of increasing amounts of 2-hydroxypropyl β-cyclodextrin.

1 μM P450 3A4 in 0.1 M KPB, 5%v/v glycerol, pH 8 at 27°C, was titrated with 2-hydroxypropyl β-cyclodextrin using method 1. Data zeroed at 450nm for presentation purposes.
Figure 4-22: Plot of $\Delta(390-418)$Absorbance vs. concentration of encapsulated testosterone for P450 3A4 (E911, Q flow through) taking into account the effect of 2-hydroxypropyl $\beta$-cyclodextrin.

i) Effect of removing the contribution of the cyclodextrin from the absorbance change.

ii) Truncated data set for determining $K_d$.

The plotted data are from one optical titration experiment. Data obtained under the same conditions as described in Figure 4-19.

Figure 4-23: Comparison of optical binding data for testosterone (TES) solubilised using either 2-hydroxypropyl $\beta$-cyclodextrin or 5% w/v DMSO.

$\Delta = 5\%$ DMSO

$\bullet = 2$-hydroxypropyl $\beta$-cyclodextrin

This figure is an overlay of data from figure 4-16 ii and figure 4-22.
Figure 4-24: UV/Vis spectral analysis of P450 3A4-cyclodextrin encapsulated testosterone complexes.

a) P450 3A4 (E911, Q, flow through) with cyclodextrin encapsulated testosterone (1750 μM).

b) Simulation of 59% high spin P450 101.

No other group has used an encapsulated form of testosterone with P450 3A4, and so no comparisons can be made.

4.2.3.1 Determining percentage high spin

By using the absolute optical spectrum data of P450 3A4 (E911, Q, flow through) with the highest concentration of encapsulated testosterone (1750 μM), a 390/418 value of 0.98 was obtained. From a simulation of P450 101 with the same 390/418 ratio a high spin content of 59% was deduced. The fact that the P450 3A4-encapsulated testosterone complex was visually similar to the simulation of P450 101 indicated that the deduced percentage high spin of the complex was a good estimate (Figure 4-24).
4.2.4 Amitriptyline; Determining $K_d$, $\Delta A_{max}$ and $n$

Optical binding studies with amitriptyline were only carried out on CHAPS solubilised protein, since it was clear that E911 inhibited substrate binding to P450 3A4. Amitriptyline appeared to be soluble in 0.1 M KPB pH 7.4 up to at least 5 mM.

Addition of amitriptyline to the CHAPS solubilised samples of P450 3A4 caused a type I optical change (Figure 4-25). Analysis of the data suggested that this substrate interacts in a cooperative way with all of the CHAPS solubilised samples of P450 3A4, since lower percentage errors on the dissociation constants were obtained when using the cooperative equation, e.g. P450 3A4 (CHAPS, HiTrap Q, 50 mM KCl) gave a $K_d$ of 175 +/- 9 (5%) with the hyperbolic equation whereas a $K_{0.5}$ of 153 +/- 4 (3%) was obtained with the cooperative equation (Figure 4-26 and Table 4-7). In addition the degree of cooperativity appears to be similar for all the samples of CHAPS solubilised P450 3A4, c.f. $n=1.25 +/- 0.04$ and $1.22 +/- 0.01$ for P450 3A4 (CHAPS, Q, 50 mM KCl) and P450 (2 mM CHAPS, S200), respectively. The data also indicated that all the fractions of P450 3A4 bound amitriptyline equally tightly, c.f. 153 +/- 4 (3%) µM and 155 +/- 7 (1%) µM for P450 3A4 (CHAPS, Q, 50 mM KCl) and P450 (2 mM CHAPS, S200), respectively.

No other group has performed equilibrium binding experiments with amitriptyline and P450 3A4, and so there are no data for comparison.
Figure 4-25: Optical difference spectra obtained with amitriptyline.

Difference spectra for the following P450 3A4 samples:

i) CHAPS, Q, 50 mM KCl

ii) 2mM CHAPS, S200

1µM P450 3A4 in 0.1M KP B, pH 7.5 at 27°C, was titrated with amitriptyline using method 1. Data zeroed at 450nm for presentation purposes. The solid arrows mark the absorbance changes upon amitriptyline addition.
Figure 4-26: Plot of $\Delta (390-418)$ Absorbance vs. concentration of amitriptyline for all protein samples purified with CHAPS.

Plots of absorbance change (390-418) against amitriptyline (AMT) concentration for the following P450 3A4 samples:

i) CHAPS, Q, 50 mM KCl (○)

ii) 2 mM CHAPS, S200 (▲)

The plotted data are from one optical titration experiment. The hyperbolic and coop NL fits are the red and blue lines, respectively.
Table 4-7: Optical binding data determined for amitriptyline.
The values obtained are for one data set, thus the standard deviations have been obtained from the fitting program.

4.2.4.1 Determining percentage high spin

By using the 390/418 values from the absolute optical spectrum data of the CHAPS solubilised P450 3A4 with the highest concentration of amitriptyline (1750 µM), simulations of P450 101 could be produced, from which a percentage high spin content was deduced for the samples of protein (Table 4-8).

<table>
<thead>
<tr>
<th>P450 3A4 sample</th>
<th>390/418</th>
<th>% High Spin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3A4</td>
<td>101</td>
</tr>
<tr>
<td>CHAPS, Q, both fractions</td>
<td>1.11</td>
<td>1.12</td>
</tr>
<tr>
<td>2 mM CHAPS, S200</td>
<td>1.57</td>
<td>1.56</td>
</tr>
</tbody>
</table>

Table 4-8: Spin state data for the P450 3A4-amitriptyline complexes.
Since a good fit was obtained visually between the P450 3A4-amitriptyline complex and the P450 101 simulation, the percentage high spin values obtained were considered to be a good estimate (Figure 4-27).

4.2.5 Conclusions from optical spectroscopy

The optical binding studies demonstrated that:

Firstly, both E911- and CHAPS-purified protein samples could bind substrates, since they induced a type I optical change, but that CHAPS-purified protein bound them more tightly.
This is mostly likely because E911 competes more effectively than CHAPS for the active site of P450 3A4, \( K_d \) of 7.2 \( \mu \)M for Triton N-101 (similar structure to E911) (Hosea and Guengerich, 1998) and 2.7 mM for CHAPS (see Chapter Five). In addition, in the case of P450 3A4 (2 mM CHAPS, S200) the interaction with testosterone was found to be cooperative, indicating that perhaps this sample of protein is the most functionally active.

Secondly, the substrates may be in fast exchange with P450 3A4, which is a pre-requisite for the NMR studies to work (see section 4.1.3.3), since all the determined \( K_d \) values were at least 100 \( \mu \)M, which indicated quite weak binding.

Thirdly, the substrates could be binding close to the haem, since they all give large shifts to high spin (except testosterone with E911-purified protein), \textit{e.g.} the P450 3A4 (2 mM CHAPS, S200)-amitriptyline complex is 79\% high spin, and therefore should produce significant relaxation rate changes (see section 4.1.3.4).

Therefore, the data suggests all of these samples of P450 3A4 should give NMR results that could be used to obtain distance data.

4.3 Results and discussion of NMR spectroscopy data

Part 1 of this section will describe and validate the development of a modified inversion recovery experiment.

Part 2 of this section will then report the results obtained with: i) testosterone, ii) encapsulated testosterone, and iii) amitriptyline.

4.3.1 Validation of new pulse program

In order to eliminate or significantly reduce the water signal from the spectrum so that weaker signals could be observed, the typical inversion recovery experiment was altered so that it contained water pre-saturation (see appendix B). As a result, the new NMR pulse program was checked to ensure that it could still be used to determine \( R_1 \) values. The \( R_1 \) values of \( \beta \)-cyclodextrin were obtained under the same conditions as reported by Amato \textit{et al} (Amato et al., 1998), who used the conventional inversion recovery experiment, and then compared to their results (Table 4-9).
Table 4-9: Comparison of published $T_1$ data for $\beta$-cyclodextrin with those obtained in the present study with a modified inversion recovery experiment.

Standard deviations have been obtained from fitting program.

<table>
<thead>
<tr>
<th>Present study</th>
<th>(Amato et al., 1998)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppm</td>
<td>$R_1$/s</td>
</tr>
<tr>
<td>5.10</td>
<td>1.08 +/- 0.03</td>
</tr>
<tr>
<td>3.99</td>
<td>0.64 +/- 0.02</td>
</tr>
<tr>
<td>3.69</td>
<td>0.67 +/- 0.02</td>
</tr>
<tr>
<td>3.61</td>
<td>1.10 +/- 0.03</td>
</tr>
</tbody>
</table>

The fact that the $R_1$ values determined in the present study were very similar to the published values strongly suggested that the pulse program was unaffected by the addition of water pre-saturation.

4.3.2 Effect of P450 3A4 on the $R_1$ values of substrate protons

4.3.2.1 Testosterone

The 1D spectrum of testosterone was obtained and assigned (Figure 4-28) using both a COSY experiment and a homonuclear decoupling experiment (data not shown).

Since P450 3A4 oxidises testosterone mainly at the 6$\beta$ position (Wang et al., 1997; Imaoka et al, 1992), this proton should approach nearest the haem iron and, therefore, should be the most sensitive to the effects of the paramagnetic centre. However, because the 6$\beta$ proton could not be unambiguously assigned, since it overlaps with the 6$\alpha$ proton and the protons on carbon 2, it could not be used. In addition, it could be seen that the spectrum had two regions where many protons had similar chemical shift and so their resonances overlapped, e.g. 1$\alpha$, 8$\beta$, 15$\alpha$ and 11$\beta$ all have multiplet structures and occur between 1.48 and 1.67 ppm, thus forming a broad set of peaks. Due to the overlapping nature of all these resonances none of them were suitable for determining distances to the iron haem. In
Figure 4-28: Assignment of 1D spectrum of testosterone.
contrast to these resonances, protons 4, 7β, 12β, 15β, and 12α, and the methyl groups 18 and 19, did not overlap with any other resonances and so could be used to investigate testosterone binding to P450 3A4.

Upon addition of E911-purified P450 3A4 to testosterone it could be seen that, although the protein concentration was very low (1 μM) more resonances appeared in the aliphatic region, which further obscured some resonances. This effect is due to E911, since characteristic resonances of this compound can be seen in the aromatic region (Figure 4-29). In contrast, no CHAPS resonances could be observed when the same amount of CHAPS-purified P450 3A4 (1 μM) was added to testosterone (Figure 4-30). This was unexpected, since theoretically CHAPS is at a higher concentration than E911 in the stock solutions of P450 3A4, cf. 2 mM CHAPS and 1.4 mM E911 (molecular weight is 705), and roughly the same volume of each protein solution has been used to produce each NMR sample. Although the reason for this is partly because E911 has more equivalent proton resonances in the aliphatic region than CHAPS, this does not explain why the CHAPS resonances cannot be seen at all. Therefore, this observation suggests that there is more E911 than CHAPS present in the respective protein stock solutions. This is entirely possible, since during the final protein concentration step E911 would not be able to travel through the pores of the Centriprep YM-30 because, as a micelle, it would be too large (E911 forms micelles of 100 or more, which would be equivalent to over 60 kDa, whereas the approximate molecular weight cut off of a Centriprep YM-30 is 30 kDa). CHAPS, however, forms much smaller micelles and these would be able to flow through the pores of this concentration device. Thus, this result suggests that both the protein and E911, but not CHAPS, are concentrated during the final concentration step, and so a greater amount of E911 is present in the NMR sample.

The R₁,obs values, determined in the presence of the various protein batches, were then obtained for proton 4 and methyl 18. These resonances were chosen because they have sharp intense bands. Although methyl 18 overlaps with the E911 resonances it is much more intense and so should still yield good data. In addition, since testosterone is oxidised mainly at the 6β position it seemed reasonable to presume that proton 4 would approach more closely to the haem iron than methyl 18 and so they would give significantly
Figure 4-29: $^1$H spectra of E911, testosterone, and testosterone in the presence of P450 3A4 purified using E911.

A = $^1$H spectrum of E911 in 0.1 M KPB pH 8.0

B = $^1$H spectrum of 0.4 mM testosterone

C = $^1$H spectrum of 1 μM P450 3A4 (E911, Q, flow through) with 0.4 mM testosterone.

B and C were dissolved in 0.1 M KPB, 5% w/v d8 glycerol, 5% w/v d6 DMSO, pH 8.0. The solid black arrows indicate the presence of E911.
Figure 4-30: $^1$H spectra of CHAPS, testosterone, and testosterone in the presence of P450 3A4 purified using CHAPS.

A = $^1$H spectrum of CHAPS in 0.1 M KP B pH 8.0

B = $^1$H spectrum of 0.4 mM testosterone

C = $^1$H spectrum of 1 μM P450 3A4 (CHAPS, Q, 50 mM KCl) with 0.4 mM testosterone.

B and C were in 0.1 M KP B, 5% v/v d₆ glycerol, 5% v/v d₆ DMSO, pH 8.0.
different $R_{1,\text{obs}}$ values. The $R_{1,\text{obs}}-R_f$ values for proton 4 and methyl 18 of testosterone, in the presence of P450 3A4 (E911, Q, flow through) and (CHAPS, Q, 50 mM KCl) were compared to each other, and to theoretically calculated $R_{1,\text{obs}}-R_f$ values for a proton at 4-10 Å from the haem iron (Figure 4-31). It should be noted that the data for both samples of E911 purified P450 3A4 was found to be almost identical, as was the data for the CHAPS purified samples of P450 3A4, and so the results for the other samples have not been reported.

From Figure 4-31, it can be seen that both E911- and CHAPS-purified protein increased the $R_{1,\text{obs}}-R_f$ rates by approximately the same amount, cf. 0.83 +/- 0.05 and 0.99 +/- 0.05 for proton 4 on testosterone for P450 3A4 (E911, Q, flow through) and P450 3A4 (CHAPS, HiTrap Q, 50 mM KCl), respectively. This was an unexpected result because these protein samples have very different $K_d$'s with testosterone. Equation 4-5 indicates that the $K_d$ only reduces the $R_{1,\text{obs}}$ value significantly when it is of a comparable size to the substrate concentration. Since the estimated $K_d$ for the E911-purified protein samples is similar to the substrate concentration used in these experiments, and five time greater than the $K_d$ determined for the batches of CHAPS-purified protein, the $R_{1,\text{obs}}$ values should be smaller for these samples of protein. However, this could be because testosterone binds to E911 micelles, which would cause it to tumble more slowly, since the micelles are much heavier than testosterone, and thus make $T_1$ relaxation more efficient (see Figure 4-8), which would result in an increase in $R_1$. Also, since CHAPS has a much higher CMC, and its micelles are much smaller it would not be expected to affect the $R_1$ values of testosterone at the concentrations present in these experiments. In order to determine the actual effect of the detergent micelles, and any similar effects brought about by binding to such a large protein as P450 3A4, it is possible to generate the diamagnetic form of the protein, i.e. the reduced-CO bound complex, and then to observe the effect it has on the substrate's $R_1$ values. However, this experiment was not carried out.

It was also observed that none of the protein samples increased the $R_{1,\text{obs}}-R_f$ values, for either proton 4 or methyl 18 on testosterone, as dramatically as would be theoretically predicted for a proton that comes into close proximity to a haem iron. These data can only be explained in one of three ways:
Chapter Four

Theoretical values

Proton 4 \( R_{\text{obs}}-R_I = 0.83 \text{ s}^{-1} \)

Methyl 18 \( R_{\text{obs}}-R_I = 0.49 \text{ s}^{-1} \)

Figure 4-31: Comparison of the \( R_{\text{obs}}-R_I \) values for protons on testosterone obtained in the presence of P450 3A4 with those determined theoretically.

Plots of \( R_{\text{obs}}-R_I \) against proton distance from the iron for the following P450 3A4 samples:

i) E911, Q, flow through.

ii) CHAPS, Q, 50 mM KCl

All \( R_{\text{obs}} \) data are for 0.4 mM testosterone in the presence of 1 \( \mu \text{M} \) P450 3A4 in the same buffer conditions as described in Figure 4-29. \( R_I \) values for proton 4 and methyl 18 were 0.48 and 1.19 s\(^{-1}\), respectively. Theoretical \( R_{\text{obs}}-R_I \) values were obtained using an \( R_I = 0.48 \text{ s}^{-1} \) and the \( K_d \) values, and effective S numbers that were determined from the optical binding experiments. In the case of E911 purified P450 3A4 a \( K_d \) of 500 \( \mu \text{M} \) was used.
1) The "active" protein concentration could have been miscalculated. Although the P450 concentration determination assay yields information on how much haem coordinated to a P450 can bind carbon monoxide (CO), this may not be the same as how much can bind substrate close to it, the "active" content, since CO is a much smaller compound. Thus, it is possible that the active content is actually less than the P450-CO concentration determined. As protein concentration decreases the $R_{1,obs} - R_f$ values for both close and distant protons will decrease and eventually converge at zero, since they cannot drop below their respective $R_{1,f}$ values. However, in the case of P450 3A4 (2 mM CHAPS, S200), if proton 4 were 4Å away from the haem iron the active protein concentration would have to be as low as 28 nM in order to obtain an $R_{1,obs} - R_f$ value of 1.2 s$^{-1}$. Since no discernable change in spin state would be observed in the optical binding studies if the active concentration was that low this explanation seems unlikely.

2) The systems may not be in fast exchange, and so the changes in the $R_{1,obs} - R_f$ values do not describe the distance that a particular proton is from the haem iron.

3) In these preparations of P450 3A4, proton 4 and methyl 18 on testosterone may actually bind further than 6Å away from the haem iron in P450 3A4. If this were the case, it would suggest that the substrate has a large amount of mobility in the active site and the value obtained is in fact an average distance, since the NMR reports on the average of all the possible substrate conformations within the active site. Alternatively, it could suggest that the protein doesn't hold the substrate in a position ready for monooxygenation in the oxidised state, which is the case in P450 102 when binding palmitoleic acid or lauric acid (Modi et al., 1996b), (Li and Poulos, 1997).

Therefore, the exchange regime of the system was investigated. Although the standard method for doing this is to obtain $R_{1,b}$ at two different temperatures, and then to compare these values to see if it has increased, slow exchange, or decreased, fast exchange, with increasing temperature, in this case it was not practical. This was because only a narrow concentration range of testosterone (0.2-0.4 mM) could be investigated, which made fitting a curve to the data very imprecise, e.g. the data for P450 3A4 (E911, Q, flow through) yielded an $R_{1,b} = 151 +/- 36$ s$^{-1}$ but a $K_D = 0.001 +/- 0.05$. The accuracy of this fit could have theoretically been improved by dropping to 0.1 mM testosterone, but in order to achieve the required signal to noise ratio for a precise $R_1$ value this would have taken a lot longer (> 3 hours), and over this period, at 27°C, protein precipitation had been observed.
However, by assuming that the $K_d$ does not change significantly with temperature, the $R_{1,obs}$-$R_f$ values can be compared in the same way as the $R_{1,b}$ would have been.

As a result, the $R_{1,obs}$-$R_f$ values for proton 4 were obtained at three different testosterone concentrations and at 10°C and 27°C (Table 4-10). The data for proton 4 is reported here, since it is representative of the results obtained for methyl 18 as well.

From Table 4-10 (i-iii) it could be seen that for all of the protein samples investigated the $R_{1,obs}$-$R_f$ values for proton 4 increased with an increase in temperature and, since the $R_{1,obs}$-$R_f$ values increase with decreasing testosterone concentration by roughly the same amount at both temperatures, it would also appear that the $K_d$ does not changed dramatically between the two temperatures. Therefore, this data suggests that all these protein samples are in slow/intermediate exchange with testosterone and thus the changes in $R_{1,obs}$ value cannot be used to determine iron haem-substrate proton distances.

4.3.2.2 2-hydroxypropyl $\beta$-cyclodextrin encapsulated testosterone

Cyclodextrin-encapsulated testosterone was used to remove the possibly deleterious effect of DMSO on E911-purified P450 3A4. In addition, if the NMR experiments worked in the presence of a cyclodextrin it would present a general methodology for studying other insoluble substrates of P450 3A4, since they could also be solubilised using these versatile encapsulating agents.

The 1D spectra of testosterone and the encapsulated form of testosterone were obtained and compared (Figure 4-32).

From this it could be seen that the chemical shift of proton 4 and methyl 18 had increased in the encapsulated form, but that their amplitude was still as great. This suggested that testosterone was in fast exchange, in terms of the chemical shift time scale, with the cyclodextrin. This was encouraging since in order for the paramagnetic relaxation experiments to work the interaction between the cyclodextrin and testosterone should not be rate limiting. In order to estimate this rate, a $K_a$ was determined for the cyclodextrin-testosterone complex by titrating free testosterone with 2-hydroxypropyl $\beta$-cyclodextrin (Figure 4-33, Table 4-11).
Table 4-10: $R_{i,\text{obs}}-R_f$ values for proton 4 on testosterone in the presence of P450 3A4 at 10°C and 27°C.

$i)$

<table>
<thead>
<tr>
<th>Substrate concentration/mM</th>
<th>10°C</th>
<th>27°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_{i,\text{obs}}-R_f$/s^{-1}</td>
<td>$R_{i,\text{obs}}-R_f^{-1}$/s</td>
</tr>
<tr>
<td>0.2</td>
<td>0.69 +/- 0.02</td>
<td>1.22 +/- 0.04</td>
</tr>
<tr>
<td>0.3</td>
<td>0.48 +/- 0.02</td>
<td>0.97 +/- 0.03</td>
</tr>
<tr>
<td>0.4</td>
<td>0.31 +/- 0.01</td>
<td>0.72 +/- 0.02</td>
</tr>
</tbody>
</table>

$ii)$

<table>
<thead>
<tr>
<th>Substrate concentration/mM</th>
<th>10°C</th>
<th>27°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_{i,\text{obs}}-R_f$/s^{-1}</td>
<td>$R_{i,\text{obs}}-R_f^{-1}$/s</td>
</tr>
<tr>
<td>0.2</td>
<td>1.11 +/- 0.04</td>
<td>1.62 +/- 0.05</td>
</tr>
<tr>
<td>0.3</td>
<td>0.81 +/- 0.03</td>
<td>1.20 +/- 0.04</td>
</tr>
<tr>
<td>0.4</td>
<td>0.50 +/- 0.02</td>
<td>0.83 +/- 0.03</td>
</tr>
</tbody>
</table>

$iii)$

<table>
<thead>
<tr>
<th>Substrate concentration/mM</th>
<th>10°C</th>
<th>27°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_{i,\text{obs}}-R_f$/s^{-1}</td>
<td>$R_{i,\text{obs}}-R_f^{-1}$/s</td>
</tr>
<tr>
<td>0.2</td>
<td>1.41 +/- 0.05</td>
<td>1.82 +/- 0.06</td>
</tr>
<tr>
<td>0.3</td>
<td>1.03 +/- 0.03</td>
<td>1.42 +/- 0.05</td>
</tr>
<tr>
<td>0.4</td>
<td>0.69 +/- 0.02</td>
<td>1.04 +/- 0.03</td>
</tr>
</tbody>
</table>

$^a$ $R_f = 0.66$ s^{-1}

$^b$ $R_f = 0.48$ s^{-1}

Standard deviations have been obtained from fitting program.

$R_{i,\text{obs}}-R_f$ data for the following P450 3A4 samples:

$i)$ E911, Q, flow through

$ii)$ CHAPS, Q, 50 mM KCl

$iii)$ 2 mM CHAPS, S200

All $R_{i,\text{obs}}$ data are for testosterone in the presence of 1 μM P450 3A4 in the same buffer conditions as described in Figure 4-29.
Figure 4-32: $^1$H spectra of testosterone and encapsulated testosterone.

A = $^1$H spectrum of 0.4 mM testosterone in 0.1 M KPB, 5% v/v $d_8$ glycerol, 5% v/v $d_6$ DMSO, pH 8.0.

B = $^1$H spectrum of 1 mM testosterone, encapsulated within 2-hydroxypropyl $\beta$-cyclodextrin, in 0.1 M KPB, 5% v/v $d_8$ glycerol pH 8.

The black arrow indicates a cyclodextrin resonance. The red arrows highlight how the chemical shifts of both proton 4 and methyl 18 have changed.
Figure 4-33: Determination of dissociation constant between cyclodextrin and testosterone.

Plots of $\Delta$(Bound-Free) in ppm against cyclodextrin concentration for proton 4 and methyl 18 on testosterone at 27°C. 0.4 mM testosterone in 0.1 M KPB, 5% v/v $d_8$ glycerol, 5% v/v $d_6$ DMSO pH 8 was titrated with two stock concentrations of cyclodextrin, which were dissolved in the same buffer.

Table 4-11: NMR binding data for testosterone with 2-hydroxypropyl $\beta$-cyclodextrin.

Dissociation constants obtained by fitting the hyperbolic equation to the data presented in Figure 4-33. Standard deviations have been obtained from fitting program.

<table>
<thead>
<tr>
<th>Resonance</th>
<th>$K_d$/μM</th>
<th>$\Delta$ppm$_{max}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proton 4</td>
<td>98 +/- 3</td>
<td>0.096 +/- 0.001</td>
<td>0.999</td>
</tr>
<tr>
<td>Methyl 18</td>
<td>116 +/- 6</td>
<td>0.078 +/- 0.001</td>
<td>0.999</td>
</tr>
</tbody>
</table>

These data strongly suggest that the change in chemical shift for both proton 4 and methyl 18 are due to the same interaction, i.e. binding to the cyclodextrin, and that the $K_d$ for this interaction is approximately 100 μM. The on rate for these two relatively small molecules, in comparison to a protein, should be approximately diffusion rate limited and of the order of $10^8$ M$^{-1}$ s$^{-1}$ (Creighton, 1993), and thus an off rate of $10^4$ s$^{-1}$ can be estimated. Although the apparent $K_d$ between the protein and the cyclodextrin encapsulated testosterone was
480 +/- 58 μM, which would suggest that this complex is weaker than the cyclodextrin-testosterone complex and thus that the off rate should be faster, it seems unlikely that the on rate will be as quick as diffusion rate limiting, since the protein will diffuse much more slowly. This would suggest that the off rate for the protein-cyclodextrin encapsulated testosterone interaction should be comparable if not slightly slower to the value for the cyclodextrin-testosterone interaction estimated above. Therefore, the cyclodextrin-testosterone interaction should not affect the exchange regime between P450 3A4 and testosterone.

Prior to adding the protein to the encapsulated testosterone solution it was found that the $R_{1,f}$ values were already different to those obtained for DMSO solubilised testosterone, cf. 1.0 and 0.5 s$^{-1}$ for proton 4 in free testosterone and encapsulated testosterone, respectively. This is probably because cyclodextrin bound testosterone will tumble more slowly, since 2-hydroxypropyl β-cyclodextrin (1430 Da) is nearly five times the molecular mass of testosterone (288 Da), which will result in $T_1$ being more efficient and thus $R_1$ increasing for this bound form. In addition, the encapsulated testosterone preparation contains more cyclodextrin than testosterone, which means that the major testosterone component in solution will be the bound form and therefore the bound $R_1$ value will predominate. For example, a 1 mM solution of encapsulated testosterone would contain 1.75 mM 2-hydroxypropyl β-cyclodextrin and since the $K_d$ is around 0.1 mM, approximately 0.89 mM of this 1 mM testosterone solution would be bound to the cyclodextrin at any one time. Therefore, in order to get a true value for $R_{1,f}$ it would be necessary to perform the experiment in the presence of the diamagnetic form of the protein.

Since the $K_d$ values for E911-purified P450 3A4 with either cyclodextrin encapsulated testosterone or DMSO solubilised testosterone should be approximately the same, as judged by the optical binding data, the changes in $R_{1,obs}$ should be very similar as well, unless the system is now in fast exchange and the $R_{1,M}$ value predominates. When P450 3A4 (E911, Q, both fractions) was added to encapsulated testosterone (Figure 4-34), even smaller changes were observed to those seen when DMSO solubilised testosterone was used, cf. 0.83 +/- 0.05 and 0.33 +/- 0.03 for DMSO and cyclodextrin encapsulated testosterone, respectively, in the presence of P450 3A4 (E911, Q, flow through). Indeed in the presence of P450 3A4 (E911, Q, 50 mM KCl) the changes were even smaller for
Figure 4-34: Comparison of the $R_{\text{f,obs}} - R_{\text{f}}$ values for protons on testosterone, encapsulated within 2-hydroxypropyl $\beta$-cyclodextrin, obtained in the presence of P450 3A4 with those determined theoretically. $R_{\text{f,obs}}$ data are for 1 mM encapsulated testosterone in the presence of 1 $\mu$M P450 3A4 (E911, Q, flow through). $R_{\text{f}}$ values for proton 4 and methyl 18 were 1.01 and 1.54 s$^{-1}$, respectively. Theoretical $R_{\text{f,obs}} - R_{\text{f}}$ values were obtained using an $R_{\text{f}} = 1.01$ s$^{-1}$ and the $K_d$ value, and effective S number that were determined from the optical binding experiments.

proton 4, the $R_{\text{f,obs}} - R_{\text{f}}$ was equal to 0.24 +/- 0.02. This suggested that the system was again in slow exchange. Since CHAPS protein proved that the reduced dissociation constant was due to E911 rather than DMSO, and a substrate with improved solubility, i.e. amitriptyline, was found, further work on cyclodextrin-encapsulated testosterone was not pursued.

4.3.2.3 Amitriptyline

Amitriptyline was chosen for further investigation in the NMR experiments because it required no solubilising agents, such as DMSO, which might affect the protein's structure. In addition, this substrate demonstrated large optical spin state changes, which should indicate that it is approaching closely to the haem iron and a higher $K_d$ than testosterone, which suggested that it might be in fast exchange. Therefore, the 1D spectrum of amitriptyline was obtained and assigned (Figure 4-35) using a COSY experiment. The
Figure 4-35: $^1H$ spectra of amitriptyline.

1D proton spectrum of 1 mM amitriptyline in 0.1 M KPB pH 7.5 at 300K and 500MHz. The site of oxidation by P450 3A4 is highlighted in red on the chemical structure of amitriptyline.
proton spectrum was well resolved, which meant that accurate $R_1$ values could be
determined for the N-methyls (protons on carbon 1), and protons 2, 3 and 4. However, the
aromatic protons (5-8 and 11-14) and protons 9 and 10, could not be assigned due to them
having overlapping resonances.

When CHAPS-purified P450 3A4 was added to amitriptyline only one particularly intense
CHAPS resonance was visible, indicating that CHAPS was at a much lower concentration
than the amitriptyline, and this did not overlap with any of the substrate's resonances
(Figure 4-36). As a result $R_{1,obs}$ values could be obtained for all the resonances described
above, but for conciseness only those for proton 4 and the N-methyls will be reported.
Since this molecule is metabolised through N-demethylation (Ueng et al., 1997) the
protons on carbon 1 should have their $R_1$ values altered the most. Therefore, it is
reasonable to assume that these protons can approach within 4Å of the haem. By making
this assumption theoretical $R_{1,obs}$ values were determined, using the same methodology as
for the previous substrates, and compared with the experimentally derived values.

For each sample of CHAPS-purified P450 3A4, the $R_{1,obs}$-$R_t$ values for the N-methyls were
unexpectedly found to be smaller than those obtained for proton 4, and than would be
theoretically predicted for a proton that was 8Å away from the haem iron, cf. 0.14 $s^{-1}$ for
P450 3A4 (2 mM CHAPS, S200) to 0.59 $s^{-1}$ for the theoretically determined value. Thus,
these data indicate that proton 4 approaches more closely than the N-methyls. The
difference is not very large, however, and may be because both N-methyl groups cannot
approach closely to the haem iron centre at the same time. Since the signal measured is for
both N-methyl groups a weighted average, due to the $r^{-6}$ relationship to $R_{1,M}$, of these two
distances will be reported in the $R_{1,obs}$ value obtained. The fact that these data suggest that
the N-methyls are so far away implies that these systems were not in fast exchange, and so
the $R_{1,b}$ values for proton 4 were determined at two different temperatures in the presence
of P450 3A4 (2 mM CHAPS, S200) (Figure 4-38) and the values compared (Table 4-12).
Proton 4 was used because it gave the greatest changes in $R_{1,obs}$ value.

Since a wider concentration range could be investigated with amitriptyline a greater
proportion of the curve could be described and thus an improved fit in comparison to the
testosterone data resulted, i.e. the standard deviations of $K_d$ were at least lower than the
Figure 4-36: $^1$H spectra of amitriptyline, CHAPS and amitriptyline in the presence of P450 3A4 (2 mM CHAPS, S200).

$A = ^1$H spectrum of 0.5 mM amitriptyline.

$B = ^1$H spectrum of 1 mM CHAPS in 0.1 M KPBr, pH 7.5.

$C = ^1$H spectrum of 0.5 mM amitriptyline in the presence of 1 μM P450 3A4 (2 mM CHAPS, S200).

$A-C$ were in 0.1 M KPBr, pH 7.5.

The black arrow indicates a CHAPS resonance in C.
**Figure 4-37:** Comparison of the \( R_{1,\text{obs}}-R_f \) values for protons on amitriptyline obtained in the presence of P450 3A4 with those determined theoretically.

Plots of \( R_{1,\text{obs}}-R_f \) against proton distance from the iron for the following P450 3A4 samples:

i) CHAPS, Q, 50 mM KCl

ii) 2 mM CHAPS, S200

All \( R_{1,\text{obs}} \) data are for 0.5 mM amitriptyline in the presence of 1 \( \mu \)M P450 3A4 in the same buffer conditions as described in Figure 4-36. \( R_f \) values for proton 4 and N-methyls were 0.69 and 0.98 s\(^{-1}\), respectively. Theoretical \( R_{1,\text{obs}}-R_f \) values were obtained using an \( R_f = 0.98 \) s\(^{-1}\) and the \( K_d \) value, and effective \( S \) number that were determined from the optical binding experiments.
Figure 4-38: Determination of $R_{1,b}$ for amitriptyline at two different temperatures in the presence of P450 3A4 (2 mM CHAPS, S200).

Plots of $R_{1,obs}$ (Proton 4) against testosterone concentration at 10°C and 27°C for P450 3A4 (2 mM CHAPS, S200).

1 μM P450 3A4 in 0.1 M KPB, pH 7.5 was used for each experiment.

<table>
<thead>
<tr>
<th>Sample of P450 3A4</th>
<th>10°C</th>
<th>27°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_b$/s$^{-1}$</td>
<td>$K_a$/μM</td>
</tr>
<tr>
<td>CHAPS, S200</td>
<td>278 +/- 38</td>
<td>115 +/- 53</td>
</tr>
</tbody>
</table>

Table 4-12: Comparing $R_{1,b}$ values for amitriptyline, in the presence of P450 3A4, at 10°C and 27°C.

Standard deviations have been obtained from fitting program.

calculated values. Although these data are still not very accurate, the standard deviation for the $K_a$ at 10°C is 46% of the value, the $R_{1,b}$ value at the higher temperature is larger than the lower temperature value even taking into account the standard deviation. This would suggest that the system is in slow/intermediate exchange and therefore no substrate-haem iron distances could be obtained from this data.
4.3.3 Conclusions from NMR spectroscopy

The NMR results demonstrated that testosterone was in slow/intermediate exchange with both E911 and CHAPS-purified P450 3A4. This was unexpected, as it was believed that E911 was causing the interaction between the protein and testosterone to be in slow/intermediate exchange. Another possible reason why the protein and substrate were not in fast exchange could have been due to protein degradation during purification and so a quicker purification method was devised, \textit{i.e.} the use of an S200 column instead of a HiTrap Q. However, this did not change the exchange regime of the system either. Therefore, it was concluded that the slow/intermediate exchange regime was intrinsic to the P450 3A4-testosterone system, and so an alternative water-soluble substrate was investigated instead.

Amitriptyline was found to have a slightly higher dissociation constant in comparison to testosterone, \textit{cf.} 100 \(\mu\text{M}\) to 150-160 \(\mu\text{M}\) for testosterone or amitriptyline, respectively, for CHAPS-purified P450 3A4-testosterone using optical spectroscopy, and thus should bind less tightly. However, amitriptyline was also found to be in slow/intermediate exchange with P450 3A4. The fact that both testosterone and amitriptyline are in slow/intermediate exchange with P450 3A4 is contrary to what would have been expected. This is because it has been reported that P450 2C9 binds diclofenac with a \(K_d\) of 25 \(\mu\text{M}\), and P450 2D6 binds codeine with a \(K_d\) of 87 \(\mu\text{M}\) and yet both these systems are in fast exchange (Poli-Scaife et al, 1997; Modi et al, 1996). The only explanation for this could be that the on and off rates for the equilibrium between P450 3A4 and substrate are slower than for these other P450s. A possible reason for this difference could be that these systems have much less detergent present. One of the most hydrophobic parts of P450 proteins is the F-G loop region (Cosme and Johnson, 2000; Williams et al, 2000), which is where the substrate access channel begins (Graham-Lorence and Peterson, 1995), therefore this area could well be covered with detergent molecules that could prevent or greatly slow down substrate entry/egress. If P450s 2C9 and 2D6 are more soluble than P450 3A4 and thus require less detergent to stabilise them in solution, then these P450s could well bind and release substrates much faster. Another possible explanation is that P450 3A4 is more aggregated than these other P450s and so will diffuse more slowly through solution in comparison, \textit{i.e.} the P450 3A4-substrate equilibrium will have a slower on rate. In addition, since the aggregation will most likely occur around the F-G loop region this could further slow the
Chapter Four

on rate and probably the off rate as well. If P450 3A4 is intrinsically less soluble than these other P450s then only a certain amount of detergent could ever be removed from the protein before precipitation occurs. In addition, the removal of detergent could actually be counter-productive since the resultant protein would be more aggregated (see chapter three section 3.2.6 for the effect of reducing CHAPS concentration on the aggregation state of P450 3A4). Therefore, it was believed that the removal of detergent from the protein preparation would not assist in producing a fast exchange regime system. This theory was confirmed by collaborators who have also attempted these NMR experiments with P450 3A4 and found their system not to be in fast exchange, since their purification procedure involved the removal of CHAPS (K. Khan, Personal Communication).

An alternative approach could have been to investigate even more weakly binding substrates (> 1 mM), but since these would probably bind in numerous different orientations and the NMR experiments would observe a weighted average of these, due to the r⁻⁶ relationship to Rᵢ,M, it was decided that this was not a desirable option. Therefore, it was concluded that the investigation into substrate binding should focus more on the optical binding experiments.

Finally, the NMR work demonstrated that 2-hydroxypropyl β-cyclodextrin bound testosterone quite weakly, which suggested that the off rate would be very quick (approximately 10⁴ s⁻¹, assuming a kᵯ of 10⁸ M⁻¹ s⁻¹). Testosterone is quite a hydrophobic molecule and so it seems reasonable to suggest that other hydrophobic molecules will be bound equally weakly. Therefore, cyclodextrin-solubilised substrates could have been used for these paramagnetic relaxation experiments with P450 3A4, if the protein-substrate interaction had been on the fast exchange time scale.
Chapter Five

Further optical binding studies with CHAPS purified P450 3A4
Further optical binding studies with CHAPS purified P450 3A4

5.1 Introduction

The results presented in this chapter constitute a preliminary study into whether any correlation exists between substrate (or analogue) or product structure and its dissociation constant, as determined by visible optical spectroscopy.

This work was performed using protein that was produced using purification method B, but where 0.5 mM CHAPS was the final detergent concentration used. The titration buffer (0.05 M KPB, 0.15 M KCl, 1 mM DTT and 1 mM EDTA pH 7.5) was chosen to reflect some of the native conditions that the protein would be exposed to, i.e. high salt concentration, mildly reducing conditions and a pH just above neutral. EDTA was added to eliminate the chance of any divalent ions from the buffer being present in solution, since P450 3A4 has been shown to be sensitive to such species. This study represents only a preliminary screen, since only single titrations have been performed on each compound.

The substrates were selected from a list that had been compiled by Guengerich (Guengerich, 1999).

5.1.1 Background

It has been suggested that the catalytic selectivity of P450 3A4 is determined more by the chemical reactivity of a substrate than by enzymatic restraints. Indeed, it has been observed that many of the monooxygenation reactions that P450 3A4 carries out are at activated positions, e.g. N-demethylation and allylic hydroxylation, thus it is has been suggested that certain types of reaction govern the selectivity of this enzyme rather than the size, shape or charge characteristics of a substrate (Smith and Jones, 1992; Stresser and Kupfer, 1997). This theory has been strengthened by the fact that many mutagenesis studies have yielded no alterations to the preferential 6β-hydroxylation of testosterone, which is an allylic position (Harlow and Halpert, 1997; He et al., 1997; Domanski et al., 1998). However, one would expect both 6β and 6α hydroxylation if the catalytic selectivity is determined by the chemical reactivity of the substrate alone, since both these
positions should be equally as reactive. In contrast to this first study, however, a further investigation demonstrated that by altering Ser-119 it was possible to make P450 3A4 a 2β- rather than 6β-steroid hydroxylase (Roussel et al., 2000). In addition, another study highlighted that certain residues within the SRSs of P450 3A4 could affect the regiospecificity towards aflatoxin B\(_1\) without influencing the metabolism of testosterone (Wang et al., 1998).

Another method for studying the nature of P450 3A4's active site would be to carry out kinetic studies with this P450 and a series of substrate analogues. If the active site was just a large cavity then the substrate would be able to rotate freely within it and small changes to the substrate structure, that do not effect its chemical reactivity, would have little effect on the regioselectivity of reaction or indeed the affinity (or \(K_{m}\)). If on the other hand the protein has a better defined active site, similar small changes to the substrate structure could lead to differences in the sites of metabolism and/or changes in the affinity. To date there has only been one such investigation into correlating metabolic specificity and affinity for P450 3A4 with substrate structure (Stresser and Kupfer, 1997). Stresser and Kupfer (Stresser and Kupfer, 1997) reported that estradiol could be ortho-hydroxylated by P450 3A4, but that estradiol 3-methyl ether could not (Figure 5-1). Also, it was found that the dissociation constants were not significantly dissimilar; \(K_{d}\) values for estradiol and estradiol 3-methyl were 270 and 370 \(\mu\)M, respectively.

Interestingly though, in the case of estradiol the \(K_{d}\) was found to be much higher than the \(K_{M}\) (15-22 \(\mu\)M) for the ortho-hydroxylation, which suggested that the spectral and kinetic parameters were reporting on different binding events. As a result, an altered binding orientation within the active site was not considered to be the explanation for the differences in metabolism. Instead, it was suggested that only the free phenolic group of estradiol could bind to the activated ferryl species to form a five membered cyclic transition state, which could then produce the ortho-hydroxylated product, i.e. the chemical reactivity of the substrate determined the final product not the enzymatic restraints. It is worth noting, however, that more recently it was shown that the \(K_{d}\) for estradiol and P450 3A4 was well correlated to the \(K_{M}\) value for ortho-hydroxylation, cf. 16 \(\mu\)M and 11 \(\mu\)M for \(K_{d}\) and \(K_{M}\), respectively (Hosea et al., 2000). This may be because Hosea \textit{et al} (Hosea et al., 2000) were using purified protein for both measurements, whereas Stresser and Kupfer
Figure 5-1: Structures of estradiol (I) and estradiol 3-methyl ether (II).
The black arrows indicate where estradiol can be ortho-hydroxylated.

(Stresser and Kupfer, 1997) used recombinant P450 3A4 expressed in insect cells for the optical binding experiments and human liver microsomes for the kinetic experiments. Alternatively, it may be because Stresser and Kupfer used different buffer conditions for these two types of experiment. Irrespective of the reason for this difference, the K_d determinations carried out by Stresser and Kupfer may be inaccurate and thus the true differences between these two substrates could have been masked. However, if both substrates were affected in the same way this may not be a point of contention.

Although an enormous amount of kinetic data for P450 3A4 and its substrates is already available (Guengerich, 1995) most of these compounds are highly dissimilar and thus any kind of correlation would be extremely difficult to make. In addition to this, the systems containing P450 3A4 which have been used are often very different, for example some studies have been carried out with human liver microsomes (Wang et al., 2000) while others have employed recombinant samples purified from E.coli that are then reconstituted with phospholipids, reductase, cytochrome b5 and synthetic detergents (Harlow and Halpert, 1998).

Carrying out an extensive kinetic study of a series of substrates and their analogues would have required a lot of time, since it would have meant performing numerous HPLC assays, and also developing new ones for compounds that have not been investigated in the past. In addition, it would not be straightforward to investigate the products of substrates since a standard would have to be obtained for the final product in order to be able to quantify the amount produced during a kinetic experiment. This would require generating a significant
amount so that it could be analysed using NMR, which may be a problem considering the fact that the product may be metabolised even more slowly than the substrate. In comparison optical binding experiments can be performed on almost any substrate/analogue, as long as it doesn't absorb too strongly within the 380-430nm range, and takes a fraction of the time. Since time was limited, it was thought that an optical binding screen performed on a series of mostly P450 3A4 substrates, but also some substrate analogues and products, would be more fruitful.

During this investigation both type I and II optical binding changes were observed and $K_d$ values were determined as described within the introduction of chapter four. In addition, however, some compounds demonstrated non-type I or II optical changes. Some evidence exists in the literature for such non-standard changes. Pirrwitz et al (Pirrwitz et al, 1982) reported that, although two spin labelled naphthalene analogues did not give rise to a typical spectral change upon addition to P450 2B4, they could still bind to the active site of this P450 because of the following facts:

1) These compounds could displace both type I and II substrates competitively  
2) Dissociation constants could be determined using EPR spectroscopy  
3) These compounds could inhibit the N-demethylation of benzphetamine

These facts suggest that the naphthalene analogues bind within the active site in such a way as to not displace the water ligand that is bound to the iron, since this is what causes the spin state change. This is equivalent to saying that these naphthalene analogues have an equal affinity for both the low and high spin forms of the protein (Ristau et al, 1978), and thus these changes have been called type 0 spectra (Pirrwitz et al, 1982).

5.2 Results and discussion

The first two parts of this section will report on the data obtained with two structurally related series of compounds (Steroids and Tricyclics), whereas the third section contains a miscellaneous set of substrates and products that have been grouped together based purely on the fact that none of them produce typical difference spectra (Miscellaneous compounds which give unusual optical binding changes).
Chapter Five

5.2.1 Steroids

The steroidal compounds investigated were CHAPS (detergent), testosterone (substrate), cortisol (substrate), cortisone (substrate analogue), 6β-hydroxytestosterone (product) and 6β-hydroxycortisone (product analogue).

It was important to determine the $K_d$ of CHAPS since it has been shown to bind to the active site of P450 3A4, and thus could potentially inhibit the binding of some substrates. In order to remove excess CHAPS from the P450 preparation, a sample of the protein stock solution was buffer exchanged into the titration buffer, containing 5% v/v glycerol, prior to its use in the binding experiments. Upon addition of CHAPS to P450 3A4 a typical type I optical change was observed (Figure 5-2 B, C). The interaction was very weak and non-cooperative since the hyperbolic equation gave the lowest percentage error on the $K_d$, cf. $K_d$ of 2734 +/- 83 μM (3%) (hyperbolic) or $K_{0.5}$ of 3379 +/- 359 μM (10%) (cooperative) (Table 5-1). Therefore, it was concluded that CHAPS should not significantly affect the binding of other compounds. It was also interesting to note that this compound resulted in an almost complete shift to high spin (Figure 5-2 B), which indicated that although this molecule binds weakly it is very effective at displacing the water.

The remainder of the steroids also gave type I difference spectra (Figure 5-3 to 5-7 C). However, it was observed that not all these difference spectra were the same in terms of the magnitude changes of the 418 nm (low spin) and 390nm (high spin) peaks (Table 5-1), and the isosbestic points. CHAPS and testosterone both gave almost symmetrical changes at 390nm and 418nm, e.g. for CHAPS the changes in absorbance were 0.089 (390) and -0.095 (418), and had an isosbestic point at approximately 405nm, whereas cortisol and cortisone gave smaller increases at 390nm, e.g. for cortisol the changes in absorbance were 0.029 (390) and -0.085 (418), and had isosbestic points at nearer 400nm. Greater changes were observed for the products, 6β-hydroxytestosterone and 6β-hydroxycortisone. These compounds produced much smaller decreases at 418nm but comparable or larger changes at 390nm, e.g. for 6β-hydroxytestosterone the absorbance changes were 0.048 (390) and -0.036 (418). Also, at higher concentrations the high spin absorbance band became broader, even when taking into account the fact that the absorbance at 450nm increased slightly during the course of the titration, thus resulting in a total loss of isosbestic point.
Figure 5-2: Optical binding data for CHAPS.

A = Structure of CHAPS

B = Absolute spectrum of P450 3A4 in absence (A) and presence of CHAPS (5253 μM) (B).

C = Difference spectra upon addition of 0-5253 μM CHAPS to P450 3A4. Numbers to the right of spectra are concentrations in μM.

D = Hyperbolic and cooperative fits to binding data (390-418).

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer was titrated with CHAPS, in the same buffer, using method 1. Protein had been buffer exchanged into titration buffer containing 5% v/v glycerol using a NAP-5 column prior to experiment.
Figure 5-3: Optical binding data for testosterone.

A = Structure of testosterone.

B = Absolute spectra of P450 3A4 in absence and presence of testosterone. Numbers to the right of spectra are concentrations in μM.

C = Difference spectra upon addition of 0-328 μM testosterone to P450 3A4. Numbers to the right of spectra are concentrations in μM.

D = Hyperbolic and cooperative fits to binding data (390-418).

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer 5% v/v DMSO was titrated with testosterone (TES), in the same buffer, using method 3.
Figure 5-4: Optical binding data for cortisol.

A = Structure of cortisol

B = Absolute spectra of P450 3A4 in absence and presence of cortisol. Numbers to the right of spectra are concentrations in μM.

C = Difference spectra upon addition of 0-899 μM cortisol to P450 3A4. Numbers to the right of spectra are concentrations in μM.

D = Hyperbolic and cooperative fits to binding data (390-418).

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer and 2% v/v DMSO was titrated with cortisol (COS), in the same buffer, using method 2.
Figure 5-5: Optical binding data for cortisone.

A = Structure of cortisone

B = Absolute spectra of P450 3A4 in absence and presence of cortisone. Numbers to the right of spectra are concentrations in µM.

C = Difference spectra upon addition of 0-612µM cortisone to P450 3A4. Numbers to the right of spectra are concentrations in µM.

D = Hyperbolic and cooperative fits to binding data (390-418).

2 µM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer and 2% v/v DMSO was titrated with cortisone (COR), in the same buffer, using method 3.
Figure 5-6: Optical binding data for 6β-hydroxytestosterone.

A = Structure of 6β-hydroxytestosterone

B = Absolute spectra of P450 3A4 in absence and presence of 6β-hydroxytestosterone. Numbers to the right of spectra are concentrations in μM.

C = Difference spectra upon addition of 0-328 μM 6β-hydroxytestosterone to P450 3A4. Numbers to the right of spectra are concentrations in μM.

D = Hyperbolic and cooperative fits to binding data (390-418).

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer and 5% v/v DMSO was titrated with 6β-hydroxytestosterone (6βOH-TES), in the same buffer, using method 3.
Figure 5-7: Optical binding data for 6β-hydroxycortisone.

A = Structure of 6β-hydroxycortisone

B = Absolute spectra of P450 3A4 in absence and presence of 6β-hydroxycortisone. Numbers to the right of spectra are concentrations in μM.

C = Difference spectra upon addition of 0-1230 μM 6β-hydroxycortisone to P450 3A4. Numbers to the right of spectra are concentrations in μM.

D = Hyperbolic and cooperative fits to binding data (390-418).

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer and 2% v/v DMSO was titrated with 6β-hydroxycortisone (6βOH-COR), in the same buffer, using method 3.
<table>
<thead>
<tr>
<th>Substrate/ Product</th>
<th>Equation</th>
<th>$K_d$ or $K_{0.5}/\mu M$</th>
<th>$\Delta A_{390}$</th>
<th>$\Delta A_{418}$</th>
<th>$\Delta A_{\text{max}}$</th>
<th>$n$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPS</td>
<td>Hyper</td>
<td>2734 +/- 83 (3%)</td>
<td>0.089</td>
<td>-0.095</td>
<td>0.289 +/- 0.004</td>
<td>n/a</td>
<td>0.998</td>
</tr>
<tr>
<td></td>
<td>Coop</td>
<td>3379 +/- 359 (10%)</td>
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<td></td>
<td>0.318 +/- 0.015</td>
<td>0.93 +/- 0.03</td>
<td>0.999</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Hyper</td>
<td>59 +/- 2 (3%)</td>
<td>0.069</td>
<td>-0.081</td>
<td>0.180 +/- 0.002</td>
<td>n/a</td>
<td>0.997</td>
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<tr>
<td></td>
<td>Coop</td>
<td>72 +/- 4 (6%)</td>
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<td></td>
<td>0.193 +/- 0.004</td>
<td>0.89 +/- 0.02</td>
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<tr>
<td>Cortisol</td>
<td>Hyper</td>
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<td>Coop</td>
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<td>0.129 +/- 0.001</td>
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</tr>
<tr>
<td>Cortisone</td>
<td>Hyper</td>
<td>105 +/- 3 (3%)</td>
<td>0.024</td>
<td>0.081</td>
<td>0.120 +/- 0.001</td>
<td>n/a</td>
<td>0.997</td>
</tr>
<tr>
<td></td>
<td>Coop</td>
<td>149 +/- 7 (5%)</td>
<td></td>
<td></td>
<td>0.137 +/- 0.002</td>
<td>0.83 +/- 0.02</td>
<td>0.999</td>
</tr>
<tr>
<td>6β-hydroxy-testosterone</td>
<td>Hyper</td>
<td>109 +/- 5 (5%)</td>
<td>0.048</td>
<td>-0.036</td>
<td>0.109 +/- 0.002</td>
<td>n/a</td>
<td>0.995</td>
</tr>
<tr>
<td></td>
<td>Coop</td>
<td>283 +/- 22 (8%)</td>
<td></td>
<td></td>
<td>0.159 +/- 0.005</td>
<td>0.74 +/- 0.01</td>
<td>0.999</td>
</tr>
<tr>
<td>6β-hydroxy-cortisone</td>
<td>Hyper</td>
<td>176 +/- 7 (4%)</td>
<td>0.033</td>
<td>-0.034</td>
<td>0.115 +/- 0.001</td>
<td>n/a</td>
<td>0.994</td>
</tr>
<tr>
<td></td>
<td>Coop</td>
<td>250 +/- 18 (7%)</td>
<td></td>
<td></td>
<td>0.131 +/- 0.003</td>
<td>0.81 +/- 0.03</td>
<td>0.998</td>
</tr>
</tbody>
</table>

**Table 5-1: Summary of binding data for steroids**

Standard deviations are from fitting program; number in brackets is the percentage error for the $K_d$ or $K_{0.5}$.

$n/a$ = non applicable

Hyper and Coop = hyperbolic and cooperative equations, respectively.
Table 5-2: Spectral properties of low spin P450 101 and when oxygen donor molecules are bound. Adapted from (Dawson et al., 1982).

These results suggest that the peak maxima or extinction coefficients of the substrate-bound high spin and low spin forms are quite sensitive to small changes in substrate structure. These changes could reflect substrate interactions with the iron, the porphyrin ring, or with amino acid residues on the protein that in turn effect the local environment of the iron. In the case of the products, the fact that they produce dissimilar difference spectra to their respective starting materials is not entirely surprising, since these compounds will most likely bind with their additional hydroxyl group close to the iron, which will be able to interact with the iron water ligand or perhaps replace it. Indeed, there are reports of alcohols forming alternative low spin complexes with P450 101, which have slightly different peak maxima and altered extinction coefficients (Table 5-2) (Dawson et al., 1982; White and Coon, 1982).

Dissociation constants were determined for all the steroids by using both the hyperbolic and the cooperative equations (Table 5-1). For all the steroids both fits gave very good correlations to the data, as demonstrated by the $R^2$ values of 0.994 and above, and so the percentage error on the dissociation constant was used as an indicator to which equation was better. Since the hyperbolic fit gave the lowest percentage error on $K_d$ for all the steroids it was concluded that none of these compounds demonstrated cooperativity towards P450 3A4. Interestingly this was contrary to the result obtained for testosterone in
Since all these systems are not cooperative the $K_d$ values, shown in brackets next to each compound, can be compared to obtain an order of affinity for P450 3A4: testosterone ($59 +/- 2 \mu M$) > cortisol ($87 +/- 1 \mu M$) > cortisone ($105 +/- 3 \mu M$) and 6β-hydroxytestosterone ($109 +/- 5 \mu M$) > 6β-hydroxycortisone ($176 +/- 4 \mu M$) >>> CHAPS ($2734 +/- 83 \mu M$). Interestingly, the order of solubility for these compounds is: testosterone ($0.4 \text{mM}$) < cortisone ($2 \text{mM}$) and cortisol ($3 \text{mM}$) < CHAPS ($15 \text{mM}$, although this is not a maximum solubility); it is worth noting that these solubilities were judged visually, *i.e.* if there was precipitate observed it was assumed that the compound was not soluble at that concentration, thus they will most likely be overestimates. Also, although a maximum stock concentration for the products was not determined, since a simple comparison to the substrate was being undertaken, these compounds will be more polar, and thus possibly more soluble than their parent compounds under these conditions. Therefore, these results could be interpreted, at least in part, in terms of the hydrophobic effect, as has been suggested in the past (Ishigami et al, 2001; Riley et al, 2001) rather than changes to the protein-substrate interactions, since it seems that as the compound becomes more polar, and thus becomes more soluble because it is able to interact with the hydrogen-bonding network of water, it has less affinity for this P450. However, the hydrophobic effect cannot be the only factor, since cortisol ($87 +/- 1 \mu M$) binds more tightly than cortisone ($105 +/- 3 \mu M$) even though cortisol ($3 \text{mM}$) is more soluble than cortisone ($2 \text{mM}$). Thus protein-substrate interactions still contribute to the observed dissociation constant. The $K_d$ determined here for testosterone ($59 +/- 2 \mu M$) is in excellent agreement with that obtained by Harlow and Halpert (Harlow and Halpert, 1998) ($56 +/- 3 \mu M$), who used the same optical binding technique. There are no literature $K_d$ values for purified P450 3A4 with any of the other compounds studied here for comparison.

### 5.2.2 Tricyclics

The common feature of the next series of compounds is that they contain two aromatic rings that are fused to either side of a seven membered ring. For brevity, this generic description has been shortened to tricyclic compounds. The tricyclic compounds studied
Figure 5-8: Optical binding data for amitriptyline.

A = Structure of amitriptyline

B = Absolute spectra of P450 3A4 upon addition of amitriptyline. Numbers to the right of spectra are concentrations of this substrate in μM.

C = Difference spectra upon addition of amitriptyline to P450 3A4. Same concentrations, and colours, as in B are used.

D = Hyperbolic and cooperative fits to binding data (390-418).

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer was titrated with amitriptyline (AMT), in the same buffer, using method 1.
**Figure 5-9: Optical binding data for clomipramine.**

A = Structure of clomipramine

B = Absolute spectra of P450 3A4 upon addition of clomipramine. Numbers to the right of spectra are concentrations of this substrate in μM.

C = Difference spectra upon addition of clomipramine to P450 3A4. Same concentrations, and colours, as in B are used.

D = Hyperbolic and cooperative fits to binding data (390-418).

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer was titrated with clomipramine (CLO), in the same buffer, using method 1.
Figure 5-10: Optical binding data for clozapine.

A = Structure of clozapine
B = Absorbance spectrum of 1 mM clozapine (350-450 nm). i = scaled to show maximum absorbance by compound. ii = scaled to the same magnitude as the absorbance changes observed in D (ii).
C = Absolute spectra of P450 3A4 upon addition of clozapine. Numbers to the right of spectra are concentrations of this substrate in μM.
D = Difference spectra upon addition of clozapine to P450 3A4. Same concentrations, and colours, as in C are used.

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer and 2% v/v DMSO was titrated with clozapine (CLZ), in the same buffer, using method 3.
Figure 5-11: Optical binding data for N-demethylated amitriptyline.

A = Structure of N-demethylated amitriptyline

B = Absolute spectra of P450 3A4 upon addition of N-demethylated amitriptyline.

Numbers to the right of spectra are concentrations of this substrate in μM.

C = Difference spectra upon addition of N-demethylated amitriptyline to P450 3A4. Same concentrations, and colours, as in B are used.

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer was titrated with N-demethylated amitriptyline (NDM-AMT), in the same buffer, using method 1.
Figure 5-12: Optical binding data for N-demethylated clozapine.
A = Structure of N-desmethyl clozapine
B = Absorbance spectrum of 800 μM N-desmethyl clozapine (350-450nm). i = scaled to show maximum absorbance by compound. ii = scaled to the same magnitude as the absorbance changes observed in D.
C = Absolute spectra of P450 3A4 upon addition of N-demethylated clozapine. Numbers to the right of spectra are concentrations of this substrate in μM.
D = Difference spectra upon addition of N-demethylated clozapine to P450 3A4. Same concentrations, and colours, as in C are used.
E = Hyperbolic and cooperative fits to binding data (425-390).
2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer and 2% v/v DMSO was titrated with N-demethylated clozapine (NDM-CLZ), in the same buffer, using method 3.
were amitriptyline (substrate), clomipramine (structurally similar to amitriptyline), clozapine (substrate), N-demethylated amitriptyline (product), and N-demethylated clozapine (product).

From the optical binding data for this series of compounds it could be seen that only amitriptyline and clomipramine gave rise to type I difference spectra (Figure 5-8 to 5-12 D). When these two data sets were analysed it could be seen that, like the steroidal data, the magnitude changes at 390 and 418 were not equal, e.g. for amitriptyline the absorbance changes were 0.108 (390nm) and -0.044 (418nm) (Table 5-3). In addition, the results showed that amitriptyline gave a maximal change at 390nm, whereas clomipramine gave its largest change at 418nm, cf. 0.039 (390nm) and -0.104 (418nm). Clozapine was found to absorb in the 350-450nm region [Figure 5-10 B (i)] and it could clearly be seen [Figure 5-10 B (ii)] that this compound would have to be added to the reference cuvette in order to blank its absorbance. At low concentrations of this compound the blanking was quite effective, but at higher concentrations (approximately 286 µM) substrate absorbance began to be significant, as a result of either experimental error or because the substrate protein complex had a larger extinction coefficient than just substrate alone (Figure 5-10 C). Between 0 and 183 µM clozapine, a very small drop in the Soret band was observed, e.g. at 183 µM substrate the minima (408nm) was -0.016, and the minima continually moved to shorter wavelength [Figure 5-10 D (i)]. From 183 to 820 µM, the end of the titration, a flattening off of the dropping Soret band between 395 and 410nm was observed, and also a small increase at 425nm, which was because of the fact that the Soret maximum had shifted from 416nm to 418nm and increased in intensity (Figure 5-10 C). These results suggest that although clozapine is not capable of displacing the iron-water ligand it is still able to modulate the coordination bond in some way, thus affecting the observed absorbance band. In addition, since two types of optical change are observed this data would support the view that there are at least two binding sites within the active site of P450 3A4 that have access to the iron and that two molecules of clozapine could bind at once, as has been proposed by other researchers (Shou et al., 1994; Korzekwa et al., 1998; Shou et al., 2001). The two types of absorbance change could arise because clozapine binds in two different orientations at each site, reflecting the fact this compound forms two products with P450 3A4, i.e. N-demethylated clozapine and an N-oxide product.
### Substrate Equation

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Equation</th>
<th>$K_d$ or $K_{o.5}/\mu M$</th>
<th>$\Delta A_{390}$</th>
<th>$\Delta A_{418}$</th>
<th>$\Delta A_{max}$</th>
<th>$n$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amitriptyline</td>
<td>Hyper</td>
<td>305 +/- 3 (1%)</td>
<td>0.108</td>
<td>-0.044</td>
<td>0.180 +/- 0.001</td>
<td>n/a</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>Coop</td>
<td>292 +/- 4 (1%)</td>
<td></td>
<td></td>
<td>0.177 +/- 0.001</td>
<td>1.04 +/- 0.01</td>
<td>0.999</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>Hyper</td>
<td>249 +/- 9 (4%)</td>
<td>0.039</td>
<td>-0.104</td>
<td>0.169 +/- 0.002</td>
<td>n/a</td>
<td>0.995</td>
</tr>
<tr>
<td></td>
<td>Coop</td>
<td>213 +/- 2 (1%)</td>
<td></td>
<td></td>
<td>0.156 +/- 0.001</td>
<td>1.26 +/- 0.02</td>
<td>0.999</td>
</tr>
<tr>
<td>Clozapine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5-3: Summary of binding data for tricyclics

Standard deviations are from fitting program; number in brackets is the percentage error for the $K_d$ or $K_{o.5}$.

$n/a$ = non applicable; Hyper, Coop = hyperbolic and cooperative equations, respectively.

( Linnet and Olesen, 1997) and at higher concentrations one orientation predominates, possibly due to multiple occupancy of the active site. Since there are at least two dissimilar optical changes occurring, and there is the overlapping effect of substrate absorbance, no attempts were made to determine a $K_d$. Interestingly, the N-demethylated product of amitriptyline did not produce a type I difference spectrum. Instead N-
demethylated amitriptyline was found to produce very similar optical difference spectra to clozapine, i.e. the two distinct types of absorbance change upon going from low (0-190 μM) to high concentrations (190-1400 μM) of this product [Figure 5-11 (i) and (ii)], and thus the same conclusions were made. Even though the data is not complicated by any additional absorbance from this product, since it does not absorb significantly in this region of the visible spectrum (data not shown), a suitable method for extracting a $K_d$ for each site has not yet been established. The other product, N-demethylated clozapine, also gave rise to difference spectra that were dissimilar to its starting material (Figure 5-12 D). In fact, it demonstrated type II optical difference spectra, which possessed an isosbestic point at approximately 417nm. Although, this product absorbs as strongly as clozapine (Figure 5-12 B), blanking seemed to be more effective, and thus no residual substrate absorbance was observed (Figure 5-12 C). The fact that substrates and their N-demethylated products have altered optical difference spectra is perhaps unsurprising since the nitrogen will be less sterically hindered and thus should be capable of coordinating to the iron to give a type II complex.

Dissociation constants were determined for amitriptyline, clomipramine and N-demethylated clozapine by fitting both the hyperbolic and cooperative equations to the data (Table 5-3). Both equations gave $R^2$ values of at least 0.987 for all three compounds thus indicating that they both fitted well to the data and so the percentage error on the dissociation constant was used as an indicator to which equation was better. The interaction between P450 3A4 and amitriptyline was non-cooperative since the cooperative equation gave no improvement in the percentage error on the dissociation constant, c.f. 305 +/- 3 (1%) and 292 +/- 4 (1%) for the hyperbolic and cooperative equations, respectively, and the $n$ value obtained was approximately 1. This once again illustrates how cooperativity is sensitive to the buffer conditions, since amitriptyline did demonstrate this type of interaction ($n = 1.22 +/- 0.01$) with P450 3A4 when using 0.1 M KPB pH 7.5. In the case of the clomipramine and N-demethylated clozapine data sets the cooperative equation yielded lower percentage errors on the $K_{0.5}$ values for and therefore suggested that these compounds interacted with P450 3A4 in a cooperative manner, c.f. for clomipramine a $K_d$ of 249 +/- 9 (4%) to a $K_{0.5}$ of 213 +/- 1% when using either the hyperbolic or cooperative equation, respectively. The fact that clomipramine interacted cooperatively ($n = 1.26 +/- 0.02$), whereas amitriptyline did not, when the differences between the two molecules is so small either suggested that the structural alterations are significant or that
the cooperative equation has just fitted to larger experimental errors that occurred with this
compound. Since the cooperative effect could be caused by the increased bulk of the
chlorine and/or the increased flexibility of the propyl side chain, further work is required to
elucidate the exact reason. N-demethylated clozapine yielded an n value of less than 1 (n =
0.71 +/- 0.01), which suggested that the interaction was negatively cooperative, i.e. two
molecules bind sequentially to the same protein, and that the second molecule binds less
tightly than the first or reduces the affinity of the first molecule for the protein. Alternatively,
this result could be obtained if two different populations of P450 3A4 existed that had dissimilar affinities for the same molecule, which has been postulated for
P450 3A4 in the past (Koley et al., 1995; Koley et al., 1997). Thus the exact reason for
this "negative" cooperativity is not known, but these results suggest that there are two
distinct sites for N-demethylated clozapine.

From the dissociation constants it could be seen that the N-demethylated product of
clozapine (50 +/- 1 μM) binds four fold tighter than clomipramine (213 +/- 2 μM), which
binds slightly tighter than amitriptyline (305 +/- 3μM) (Table 5-3). It seems likely that N-
demethylated clozapine binds tighter than the other two substrates because as well as
having the tricyclic hydrophobic moiety with which to bind to the active site, it can
coordinate to the iron through the exposed nitrogen atom. There are no literature K_d values
for purified P450 3A4 with any of the compounds studied here for comparison.

5.2.3 Miscellaneous compounds which give unusual optical binding changes

The following compounds were all found to produce non-standard difference spectra:
diltiazem (substrate), acetaminophen (substrate), dextromethorphan (substrate), codeine
(substrate), N-demethylated codeine (product), quinidine (substrate) and 3-
hydroxyquinidine (product) (Figure 5-13 to 5-20).

Although diltiazem was found to produce type I-like difference spectra at low
concentrations (0-201 μM) at higher concentration (201-1425 μM) no increase at 390nm
occurred and only a decrease at approximately 414nm resulted [Figure 5-13 C (i) and (ii)].
These results could suggest that this compound initially binds to a site where water can be
displaced from the iron, and which is very rapidly saturated, and then further substrate
molecules can only modulate the iron-water ligand interaction in some way. Given the
Figure 5-13: Optical binding data for diltiazem.

A = Structure of diltiazem

B = Absolute spectra of P450 3A4 upon addition of diltiazem. Numbers to the right of spectra are concentrations of this substrate in μM.

C = Difference spectra upon addition of diltiazem to P450 3A4. Numbers within the spectra are concentrations of this substrate in μM.

D = Hyperbolic and cooperative fits to binding data (414-450nm).

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer was titrated with diltiazem (DIL), in the same buffer, using method 1.
Figure 5-14: Optical binding data for dextromethorphan.

A = Structure of dextromethorphan

B = Absolute spectra of P450 3A4 upon addition of dextromethorphan. Numbers to the right of spectra are concentrations of this substrate in μM.

C = Difference spectra upon addition of dextromethorphan to P450 3A4. Numbers to the right of spectra are concentrations of this substrate in μM.

D = Hyperbolic and cooperative fits to binding data (414-450nm).

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer was titrated with dextromethorphan (DEX), in the same buffer, using method 1.
Figure 5-15: Optical binding data for codeine.

A = Structure of codeine
B = Absolute spectra of P450 3A4 upon addition of codeine. Numbers to the right of spectra are concentrations of this substrate in μM.
C = Difference spectra upon addition of codeine to P450 3A4. Numbers to the right of spectra are concentrations of this substrate in μM.
D = Hyperbolic and cooperative fits to binding data (414-450nm).

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer was titrated with codeine (COD), in the same buffer, using method 1.
Figure 5-16: Optical binding data for N-demethylated codeine.

A = Structure of N-demethylated codeine

B = Absolute spectra of P450 3A4 upon addition of N-demethylated codeine. Numbers to the right of spectra are concentrations of this substrate in μM.

C = Difference spectra upon addition of N-demethylated codeine to P450 3A4. Numbers to the right of spectra are concentrations of this substrate in μM.

D = Hyperbolic and cooperative fits to binding data (414-450nm).

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer and 2% v/v DMSO was titrated with N-demethylated codeine (NDM-COD), in the same buffer, using method 3.
Figure 5-17: Optical binding data for acetaminophen.

A = Structure of acetaminophen.

B = Absolute spectra of P450 3A4 upon addition of acetaminophen. Numbers to the right of spectra are concentrations of this substrate in μM.

C = Difference spectra upon addition of acetaminophen to P450 3A4. Numbers to the right of spectra are concentrations of this substrate in μM.

D = Hyperbolic and cooperative fits to binding data (414-450nm).

2 μM P450 3A4 (0.5 mM CHAPS, S200) in 0.05 M KPB, 0.15 M KCl, 1 mM DTT, 1 mM EDTA, pH 7.5 (titration buffer) was titrated with acetaminophen (ACE), in the same buffer, using method 1.
Figure 5-18: Optical binding data for quinidine.

A = Structure of quinidine

B = Absorbance spectrum of 1 mM quinidine (350-450 nm).  
   i = scaled to show maximum absorbance by compound.  ii = scaled to the same magnitude as the absorbance changes observed in D.

C = Absolute spectra of P450 3A4 upon addition of quinidine. Numbers to the right of spectra are concentrations of this substrate in µM.

D = Difference spectra upon addition of quinidine to P450 3A4.  i = 0-286 µM, ii = 286-1015 µM quinidine.

2 µM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer and 2% v/v DMSO was titrated with quinidine (QUI), in the same buffer, using method 2.
Figure 5-19: Optical binding data for 3-hydroxyquinidine.

A = Structure of 3-hydroxyquinidine

B = Absorbance spectrum of 1 mM 3-hydroxyquinidine (350-450nm). i = scaled to show maximum absorbance by compound. ii = scaled to the same magnitude as the absorbance changes observed in D.

C = Absolute spectra of P450 3A4 upon addition of 3-hydroxyquinidine. Numbers above the spectra are concentrations of this substrate in μM.

D = Difference spectra observed upon addition of 3-hydroxyquinidine to P450 3A4. The same concentrations, and colours, as in B are used. i = 0-286 μM, ii = 286-1015 μM.

2 μM P450 3A4 (0.5 mM CHAPS, S200) in titration buffer and 2% v/v DMSO was titrated with 3-hydroxyquinidine (3OH-QUI), in the same buffer, using method 2.
many oxygen donor groups available on this molecule, perhaps one or all of these can coordinate to the iron instead of the water, since it has been reported ketones, esters, ethers and carboxylic acids (in addition to alcohols) can coordinate to the iron of P450 101 (Dawson et al., 1982). These oxygen donor molecules were all found to give low spin spectra with P450 101, but with slightly different Soret maxima and extinction coefficients (Table 5-2).

Dextromethorphan, codeine and N-demethylated codeine all produced small drops in absorbance at 414nm (Figure 5-14 to 5-17), the greatest change being -0.04 for 1751 μM dextromethorphan. It is worth noting that Yamazaki et al (Yamazaki et al., 1995) also observed a small drop (ΔA\text{max} (417nm) = 0.01/μM P450) in the Soret band when studying the interaction of ethylmorphine, a structural analogue of codeine, with purified P450 3A4. It was also found for these compounds that the absorbance increased slightly either side of the minima. These results may again suggest that oxygen donors on these molecules are interacting with the haem iron in some way. Interestingly, unlike the N-demethylated product of clozapine, the N-demethylated product of codeine did not produce any kind of type II complex, which indicates that this nitrogen cannot coordinate with the iron. If the active site of P450 3A4 was just a large cavity then either of these product's nitrogens should be able to coordinate to the iron, since they both form part of a six membered aliphatic ring. These results could suggest that the bulkier nature of N-demethylated codeine, in comparison to the mainly flat N-demethylated clozapine, prevents this nitrogen from approaching at the right angle and closely enough to coordinate with the iron. Acetaminophen also produced a small overall drop (0.024) in the Soret band with a minimum at approximately 414nm, but that was broader than for dextromethorphan and its structurally related compounds (Figure 5-17 C). This probably reflects the fact that the oxygen donors on this molecule (aromatic alcohol and amide) are very different to those on dextromethorphan, codeine and N-demethylated codeine (aromatic ether, aliphatic cyclic ether, and aliphatic alcohol). Quinidine produces two dissimilar types of difference spectrum [Figure 5-18 D (i) and (ii)]. At low concentrations (0-143 μM) a very small and broad drop in the Soret band is observed with a minimum at 410nm, but at higher concentrations (143- 1015 μM) the minimum changes to approximately 390nm and a small increase at 422nm occurs. These results indicate that two distinct types of interaction
Table 5-4: Summary of binding data for the miscellaneous series of compounds

<table>
<thead>
<tr>
<th>Substrate/Product</th>
<th>Equation</th>
<th>$K_d$ or $K_{0.5}/\mu$M</th>
<th>$\Delta A_{max}$</th>
<th>n/a</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diltiazem</td>
<td>Hyper</td>
<td>283 +/- 10 (4%)</td>
<td>-0.071 +/- 0.001</td>
<td>n/a</td>
<td>0.996</td>
</tr>
<tr>
<td></td>
<td>Coop</td>
<td>465 +/- 21 (5%)</td>
<td>-0.086 +/- 0.001</td>
<td>0.78 +/- 0.01</td>
<td>0.999</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>Hyper</td>
<td>490 +/- 24 (5%)</td>
<td>-0.031 +/- 0.001</td>
<td>n/a</td>
<td>0.993</td>
</tr>
<tr>
<td></td>
<td>Coop</td>
<td>1274 +/- 160 (13%)</td>
<td>-0.044 +/- 0.002</td>
<td>0.72 +/- 0.02</td>
<td>0.998</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>Hyper</td>
<td>610 +/- 59 (10%)</td>
<td>-0.048 +/- 0.002</td>
<td>n/a</td>
<td>0.975</td>
</tr>
<tr>
<td></td>
<td>Coop</td>
<td>6000 +/- 4439 (74%)</td>
<td>-0.116 +/- 0.03</td>
<td>0.61 +/- 0.04</td>
<td>0.995</td>
</tr>
<tr>
<td>Codeine</td>
<td>Hyper</td>
<td>583 +/- 70 (12%)</td>
<td>-0.041 +/- 0.002</td>
<td>n/a</td>
<td>0.960</td>
</tr>
<tr>
<td></td>
<td>Coop</td>
<td>11676 +/- 16407 (140%)</td>
<td>-0.128 +/- 0.067</td>
<td>0.57 +/- 0.06</td>
<td>0.990</td>
</tr>
<tr>
<td>N-demethylated-</td>
<td>Hyper</td>
<td>63 +/- 6 (10%)</td>
<td>-0.020 +/- 0.001</td>
<td>n/a</td>
<td>0.963</td>
</tr>
<tr>
<td>codeine</td>
<td>Coop</td>
<td>61 +/- 5 (8%)</td>
<td>-0.019 +/- 0.001</td>
<td>1.13</td>
<td>0.964</td>
</tr>
</tbody>
</table>

Table 5-4: Summary of binding data for the miscellaneous series of compounds

Standard deviations are from fitting program; number in brackets is the percentage error for the $K_d$ or $K_{0.5}$.

$n/a = non$ applicable; Hyper and Coop = hyperbolic and cooperative equations, respectively.

occur with the iron. The first interaction is probably brought about by one of the oxygen donors that quinidine possesses coordinating to the iron (either the aromatic ether or the aliphatic alcohol), and the second may be due to one of the nitrogen donors coordinating instead (either the bicyclic bridge nitrogen or the aromatic nitrogen). The product, 3-
hydroxyquinidine, also gives rise to two dissimilar types of difference spectrum [Figure 5-19 D (i) and (ii)]. At low concentrations (0-143 μM) the changes are similar to those observed for quinidine, but at higher concentrations (143-1015 μM) although the minimum shifts to shorter wavelength (393nm) there is no increase at 422nm. This result may be because the iron preferentially interacts with the additional hydroxyl group, since it will probably still bind quite close to the iron, rather than the nitrogen donors on this compound. Since both quinidine and 3-hydroxyquinidine produce two dissimilar types of difference spectrum, $K_d$ values could not be determined for these compounds.

Dissociation constants were determined for the miscellaneous series of compounds by fitting both the hyperbolic and cooperative equations to the data (Table 5-4). The hyperbolic equation fitted well to both the diltiazem and acetaminophen data sets, i.e. the decrease in absorbance at 414nm with respect to 450nm, since the $R^2$ values were 0.996 and 0.993 for diltiazem and acetaminophen, respectively. When the cooperative equation was applied to these data sets no improvement in the percentage error of the dissociation constant was obtained and thus the interaction was not believed to be cooperative. In the case of acetaminophen this is surprising considering its small size, however, the magnitude change is quite small in comparison to the interactions observed for the steroids and tricyclics, cf. 0.18 for testosterone and 0.024 for acetaminophen, thus being able to distinguish the changes from a hyperbolic curve that indicate cooperativity to those that are merely due to experimental error becomes more difficult. In addition, the cooperative equation yields a worse percentage error than the hyperbolic equation for the dissociation constant mainly because of the extrapolated $\Delta A_{max}$ value. Firstly, the $\Delta A_{max}$ value is 42% greater than that obtained for the hyperbolic equation, cf. -0.031 (hyperbolic) and -0.044 (cooperative), which results in the dissociation constant being a lot larger, but more importantly the error associated with this value is larger, cf. -0.031 +/- 0.001 (3% error) for hyperbolic and -0.044 +/- 0.002 (5% error), which will increase the range of possible dissociation constant values. The $n$ value and its standard deviation also effect the dissociation constant and its standard deviation, but in this case not as significantly as the $\Delta A_{max}$ value. The hyperbolic fits to the dextromethorphan and codeine data sets, i.e. the decrease in absorbance at 414nm with respect to 450nm, are quite poor in comparison to the fits determined for diltiazem and acetaminophen; the $R^2$ values are 0.975 and 0.96 for dextromethorphan and codeine, respectively. Although an improvement in $R^2$ is obtained by fitting the cooperative equation to the dextromethorphan ($R^2 = 0.995$) and codeine ($R^2 =$
0.990) data sets, the percentage error on the dissociation constants has significantly increased, for example, the $K_{0.5}$ for codeine is $11676 +/- 16407 \, \mu M$ (140%), which suggests that this equation is not appropriate. These large errors are again because the $\Delta A_{\text{max}}$ values have become significantly greater, for example cf. -0.041 with -0.128 (212% increase) for codeine, and the percentage error on the $\Delta A_{\text{max}}$ has also increased dramatically, for example cf. -0.041 +/- 0.002 (5%) for the hyperbolic equation and -0.128 +/- 0.067 (52%) for the cooperative equation for the codeine data set. The standard deviations on the dissociation constants for dextromethorphan and codeine are also increased by the large errors on the $n$ values obtained, for example codeine has an $n$ value of 0.57 +/- 0.06, which is an error of 11%. Thus neither of these equations gives accurate dissociation constants for dextromethorphan or codeine and so the values obtained will not be compared to those obtained for other compounds. By examining the fits for both these data sets (Figure 5-14 D and Figure 5-15 D) it can be seen that the absorbance at 415 nm drops sharply up to around 250 $\mu M$ and then instead of the absorbance change saturating it continues to drop but more gradually. This suggests that there are two or more separate interactions occurring with very different dissociation constants, which is why the hyperbolic equation cannot fit the data properly and the cooperative fit gives a very large dissociation constant with an enormous error. One explanation for this result could be that two molecules bind sequentially to the same protein, and that the second molecule binds less tightly than the first or reduces the affinity of the first molecule for the protein. Alternatively, this result could be obtained if two different populations of P450 3A4 existed that had dissimilar affinities for the same molecule, which has been postulated for P450 3A4 in the past (Koley et al., 1995; Koley et al., 1997). Interestingly, Yamazaki et al (Yamazaki et al, 1995) found that ethylmorphine produced a drop in the Soret band ($\Delta A_{\text{max}}$ (417nm) = 0.01/$\mu M$ P450) with purified P450 3A4 as well, and reported a dissociation constant of 49 +/- 9 (18%), which is even less accurate than the value determined for codeine using the hyperbolic equation in this study cf. 583 +/- 70 (12%). In the case of the N-demethylated codeine data set, i.e. the decrease in absorbance at 414nm with respect to 450nm, neither equation can describe the data properly, hence the low $R^2$ values obtained (0.963 and 0.964 for the hyperbolic and cooperative equations, respectively) and the poor visual fits (Figure 5-16 D). As a result, the $K_d$ reported for this compound is just a number generated by the fit and so will not be used for comparison purposes. The major problem with analysing the data for dextromethorphan, codeine and N-demethylated codeine is the fact that the overall absorbance changes measured are substantially smaller in comparison
to that obtained for the steroids and tricyclics, cf. 0.035 or 0.02 for dextromethorphan and N-demethylated codeine, respectively, with 0.18 for both testosterone and amitriptyline. Thus, small experimental errors become magnified and perturbations of the solution due to slight precipitation of substrate at higher concentrations can affect the overall result more significantly. An additional complication in the interpretation of these results is that the types of optical changes observed are non-standard.

The dissociation constants suggest acetaminophen binds slightly more weakly than diltiazem to P450 3A4, cf. 490 +/- 24 µM and 283 +/- 10 µM for acetaminophen and diltiazem, respectively, which may well be because diltiazem has a greater number of hydrophobic groups with which to interact with P450 3A4, cf. Figure 5-13A with Figure 5-17A. There are no literature values of K_d for purified P450 3A4 with diltiazem or acetaminophen to compare with.

5.3 Conclusions

From this preliminary investigation it is apparent that there is a correlation between a compound's affinity for P450 3A4 and its solubility (Table 5-5), i.e. the more soluble the substrate the weaker it will bind to this protein, as has been suggested in the past (Ishigami et al, 2001; Riley et al, 2001). Thus, low solubility steroids, which require DMSO as a co-solvent to dissolve in an aqueous environment, bind tighter than most other compounds, followed by the more polar hydroxylated steroids. After the steroids, the next highest affinity compounds are the fairly aqueous soluble compounds acetaminophen, diltiazem, amitriptyline and clomipramine, which will all dissolve up to about 5 mM. Finally, the highly water-soluble compound CHAPS binds the least strongly. This correlation can be explained in terms of the hydrophobic effect. When a hydrophobic compound is in aqueous solution water molecules create a cavity for it by ordering themselves around it. When the compound binds to the protein, water molecules become less ordered, in comparison, and thus entropy increases which is thermodynamically favourable. In addition, the active site of P450 3A4, which is made up of mainly hydrophobic residues (Szklarz and Halpert, 1997), is desolvated upon substrate binding and this will also lead to an entropy increase. This will lead to a decrease in free energy, which is equivalent to a decrease in K_d. The fact that N-demethylated clozapine binds slightly more tightly to P450
<table>
<thead>
<tr>
<th>Rank order</th>
<th>Affinity (Dissociation constant)</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NDM-CLZ (50 +/- 1 μM)</td>
<td>testosterone and 6β-OH TES (0.4 mM in 5% DMSO)</td>
</tr>
<tr>
<td>2</td>
<td>testosterone (59 +/- 2 μM)</td>
<td>clozapine and NDM-CLZ (1 mM in 2% DMSO)</td>
</tr>
<tr>
<td>3</td>
<td>cortisol (87 +/- 1 μM)</td>
<td>cortisone and 6β-OH COR (2 mM in 2% DMSO)</td>
</tr>
<tr>
<td>4</td>
<td>cortisone (105 +/- 3 μM)</td>
<td>cortisol (3 mM in 2% DMSO)</td>
</tr>
<tr>
<td>5</td>
<td>6β-OH TES (109 +/- 5 μM)</td>
<td>acetaminophen, amitriptyline, clomipramine, diltiazem (5 mM)</td>
</tr>
<tr>
<td>6</td>
<td>6β-OH COR (176 +/- 7 μM)</td>
<td>CHAPS (15 μM)</td>
</tr>
<tr>
<td>7</td>
<td>clomipramine (213 +/- 2 μM)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>diltiazem (283 +/- 10 μM)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>amitriptyline (305 +/- 3 μM)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>acetaminophen (490 +/- 24 μM)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>CHAPS (2734 +/- 83 μM)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5-5: Rank order of each compounds affinity for P450 3A4 and its solubility in the buffer system used.

NDM-CLZ = N-demethylated clozapine
6β-OH TES = 6β-hydroxylated testosterone
6β-OH COR = 6β-hydroxycortisone

"CHAPS is soluble to at least 163 mM in water, since this was the stock solution prepared for the solubilisation stage of P450 3A4, see Chapter two."
3A4 than testosterone could be because a greater concentration of DMSO was used to solubilise the steroid. Alternatively, the coordination bond formed between the nitrogen of N-demethylated clozapine and the iron, which gives rise to the type II interaction, could increase the affinity of the compound for P450 3A4 by reducing the enthalpy of the system, which is thermodynamically favourable and so would result in a decrease in free energy. Another anomalous result to the theory that the hydrophobic effect predominates is that cortisol binds more tightly to P450 3A4 than cortisone, since cortisol is more soluble than cortisone. An explanation for this observation could be that the hydroxyl group participates in a hydrogen bonding interaction, which the keto group could not do, with part of the active site of P450 3A4.

This study has also indicated that P450 3A4 may impose some constraints on the substrate, thus suggesting that it may not simply possess a large cavity as an active site. Evidence for this is the fact that CHAPS, testosterone and amitriptyline, which are mainly flat molecules, all give strong type I interactions, indicating that they are able to displace the water bound to the iron very effectively, whereas clozapine, diltiazem, codeine and quinidine, which rather than being flat are more spherically shaped, do not produce either large or type I optical changes. Although the lack of a significant optical change brought about by clozapine, diltiazem or codeine could be because they interact in a type 0 way, i.e. they have an equal affinity for the water bound and water free P450, this seems unlikely since these compounds are more bulky than their type I counter parts and do not possess any greater number of polar groups that could interact with the water and thus stabilise the water-bound P450 form. One further piece of evidence for enzymatic restraints on the substrate is the observation that N-demethylated clozapine is able to interact with the iron to produce a type II optical change whereas N-demethylated codeine, which also possesses a secondary amine within a six membered ring, is not. However, the implications of these results are unclear since the oxidised and water bound P450 3A4 is unlikely to be identical to the activated oxygen intermediate that actually performs the oxidation reaction. Thus, it is difficult to comment on whether the catalytic activity of P450 3A4 is solely determined by chemical reactivity as has been previously proposed (Smith and Jones, 1992; Stresser and Kupfer, 1997), or is also dependent on enzymatic restraints.

This study also suggests that multiple binding sites may exist within P450 3A4 that could be simultaneously occupied (Shou et al., 1994; Korzekwa et al., 1998; Shou et al., 2001)
and/or that multiple conformationally different populations of P450 3A4 exist (Koley et al., 1995; Koley et al., 1997). The fact that some compounds can produce type I changes and others do not could suggest that there are at least two binding sites; one where the water can be displaced and a second where the water ligation is either modulated or possibly replaced by an oxygen on the compound itself. Although whether these are on the same molecule of P450 or on separate ones cannot be determined. The data obtained for clozapine, N-demethylated amitriptyline, quinidine and 3-hydroxylated quinidine also may suggest the existence of at least two binding sites, which may or may not be on the same P450 molecule, but also the possibility of multiple occupancy, since two distinct optical changes were obtained at different concentrations of the compound. Also, the fact that neither the hyperbolic or cooperative equations fitted well to the dextromethorphan, codeine and N-demethylated codeine data sets could indicate that two or more distinct sites or conformations of P450 3A4 exist that have dissimilar dissociation constants for these molecules. Finally, the data for clomipramine and N-demethylated clozapine suggested positive and negative cooperativity, respectively, which could be explained by multiple occupancy of the active site, although this may also result if there is an allosteric site on the surface of the protein. Thus, the main question is whether (i) a single P450 3A4 conformer exists with multiple binding sites or (ii) P450 3A4 exists as an ensemble of conformers comprising a range of structurally distinct active sites. Unfortunately, these scenarios cannot be distinguished between with data from the current study.
CHAPTER SIX

Evaluation of the present study
Chapter Six

Evaluation of the present study

6.1 Overall conclusions from this investigation

The main object of this study was to obtain haem iron-substrate proton distance data to further develop the in-house model of P450 3A4 utilising NMR. However, this objective could not be achieved, most probably because the on and off rates for substrate binding were not sufficiently fast for the purposes of NMR paramagnetic R₁ studies. This result was despite the fact that P450 3A4 could be highly purified using both E911 and CHAPS as solubilising agents and was shown to be active, as judged by the ability of the protein to bind carbon monoxide, turnover 7-BQ, and produce spin state changes with substrates, in the presence of both these detergents. The fact that the CHAPS-purified samples of protein did not produce any successful NMR experiments was surprising, since during the study it was found that these samples of P450 3A4 were significantly more active than E911-purified protein in terms of its ability to turnover 7-BQ and bind substrates. However, investigations into the aggregation state of CHAPS-purified P450 3A4, which were not carried out for E911-purified samples, showed that the P450 3A4-CHAPS complex was either a tetramer or pentamer when in the presence of either 2 or 0.5 mM CHAPS, respectively, which suggested a reason for why the on and off rates for substrate binding may have been too slow for the NMR experiments.

By utilising the optical changes observed upon substrate binding it has been possible to determine the dissociation constant between P450 3A4 and many of its substrates and products, and also to infer information about the binding events occurring with this protein. From this work a correlation between a compound's affinity for P450 3A4 and its solubility has been made, i.e. the more soluble the substrate the weaker it will bind to this protein, as has been suggested in the past (Ishigami et al, 2001; Riley et al, 2001). These studies have also indicated that, (i) the affinity of a substrate may be enhanced by the presence of a nitrogen that can coordinate to the iron, (ii) the active site is unlikely to be merely a large cavity where substrate's can bind, (iii) the cooperative behaviour of testosterone and amitriptyline with P450 3A4 is dependent upon the buffer conditions used and (iv) one or more substrate molecules may be interacting with this protein at once and at distinct binding sites, although the nature of these sites cannot be determined.
6.2 Future Work

6.2.1 Optical binding studies

In order to investigate the affinities of substrates that did not produce a typical spin state change, e.g. dextromethorphan and quinidine, a competition assay could be performed using a large weakly binding type II ligand, such as one of the peptides mentioned above. By using a large ligand it is more likely that the substrate will displace it from the active site completely rather than binding alongside it, which is a possibility given the nature of P450 3A4.

Additional work could probe alternative buffer conditions, to see how salt and pH effect both binding affinity and cooperativity of the system. Also, it would be interesting to perform these experiments under reducing conditions to see if there are any differences between the reduced and oxidised protein.

6.2.2 Protein purification and NMR

If it is assumed that it is possible to obtain an NMR active sample of P450 3A4, i.e. one that yields distance data, as has been suggested in the past (Modi, 1996, unpublished results), then a protocol to achieve this still needs to be established. The following alterations to the initial conditions, i.e. method A1 (Section 2.2.1.5), have already been tried:

i) Introduction of more protease inhibitors, such as aprotonin, leupeptin and benzamidine (see Appendix C).

ii) Less sonication (see Appendix C), because this may reduce protein sheering.

iii) Use of Tris buffer instead of KPB (see Appendix C), because the protein may be more soluble/stable in this buffer.

iv) Removal of E911 from protein sample (see Appendix C), because this detergent inhibits P450 3A4.

v) Maintaining high ionic strength at HiTrap Q step (see Appendix C), because this may generate a more stable/less aggregated protein.

vi) Using different glycerol concentrations and pHs at HiTrap Q stage (see Appendix C), since these conditions may be important in protein stability.
vii) Using CHAPS as an alternative detergent, since this inhibits P450 3A4 less than E911.

viii) Using a size-exclusion column instead of HiTrap Q, because this column can purify and buffer exchange the sample of P450 3A4 to the same extent but more quickly, thus avoiding any possible protein degradation.

In addition, further changes have been made by our collaborators in Dundee, which are as follows:

i) E913 as an alternative detergent to E911

ii) An hydroxyapatite column instead of a HiTrap Q column

iii) Adding nickel agarose resin at the same time as adding the detergent (E913). Then centrifuging the solution and collecting the resin, putting into an empty column and washing and eluting using imidazole in the usual way.

In order to obtain a system that is in fast exchange for the purposes of NMR, it may be necessary to obtain a monomeric sample of the protein. Thus future work could focus on determining the conditions required to achieve this. However, it seems likely that high concentrations of detergent will be required, as has been seen for other P450s (Kempf et al., 1995; Viner et al., 1995; Pernecky et al., 1995), and this could result in (i) inhibition of the enzyme and/or (ii) the substrate's $^1$H resonances being obscured by the surfactant. Therefore, perhaps a similar method to that adopted with P450 2C5 could be used, i.e. removal of the N-terminal hydrophobic region and mutation of relevant residues from the FG loop (Williams et al., 2000; Cosme and Johnson, 2000). However, although this may lead to monomeric protein it may not function as wild type, given the proposed role of the FG loop in forming part of the substrate access channel.

Alternatively, weakly binding compounds that produce a type II optical change could be investigated, since these would produce a low spin iron that would give rise to a smaller R$_{1,M}$ value, which would mean that the exchange rate is competing with a slower rate and so the system may move into the fast exchange regime. Although in this study it was found that nitrogen coordination increased affinity, weakly nitrogen coordinating peptides have been reported by Hosea et al (Hosea et al., 2000) with K$_d$ values of 300-470 µM, but this may be because they are more water soluble, i.e. reduced entropic driving force.
REFERENCES
References


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Graham SE and Peterson JA (1999) How similar are P450s and what can their differences teach us? Archives of biochemistry and biophysics 369:24-29.


Klingenberg M (1958) Pigments of rat liver microsomes. *Archives of biochemistry and biophysics* **75**:376-386.


substrate models: Evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry* **37**:4137-4147.


APPENDIX A
Appendix A

P450 3A4 amino acid sequence and % composition, calculated molecular weight and theoretical pI value

Amino acid sequence (503):
MALIPDLAME TWLLAVSLVLYYLYGTHSH GLFKKLGIPG PTPLPFLGNI
LSYHKGFCMF DMECHKKYGKVWGFYDGQQPVLAITDPMIDymes: VFTNRPPFGPVGMFMKSAISIAEDEEWKRLLSSPFTSG
KLKEMVPIIA QYGDLVVRNL RREAETKPV TLKDVFQAGYS MDVITSTSG
VNIDSNQPDPFVENTKDLRFDFFPLFLSITVFPIPILEVLNICVFPREVTNLR
KSKRMKESRLEDTQHRVDFLQLMIDSDQNSKETESHKAL
STDELSQASI IFIFAGYETTSSVLSFIMYELATHPDVQKLQEEIDAVLP
NKAPPTYDTVLQMEYLMVVNETLRFPIAMRLERVCKKDLVIEINGMIFIK
GWVVMIPSVAYLHRDPKYWTEPEKFLPERFSKKNKDNIDPYIYTPFGSGPR
NCIGMRFALMNMLLALIRVLQNFSFKPCKE TQIPLKLSGLGLIQPEKPVV
LKVESRDGTGTVSGA

Calculated weight: 60177.7
Theoretical pI value: 8.77

Amino acid composition:

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<tr>
<th>Non-Polar</th>
<th>No. (%)</th>
<th>Polar</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Alanine)</td>
<td>28 (5.28)</td>
<td>G (Glycine)</td>
<td>27 (5.09)</td>
</tr>
<tr>
<td>V (Valine)</td>
<td>41 (7.74)</td>
<td>S (Serine)</td>
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<tr>
<td>L (Leucine)</td>
<td>60 (11.32)</td>
<td>T (Threonine)</td>
<td>30 (5.66)</td>
</tr>
<tr>
<td>I (Isoleucine)</td>
<td>31 (5.85)</td>
<td>C (Cysteine)</td>
<td>7 (1.32)</td>
</tr>
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<td>P (Proline)</td>
<td>35 (6.6)</td>
<td>Y (Tyrosine)</td>
<td>16 (3.02)</td>
</tr>
<tr>
<td>M (Methionine)</td>
<td>20 (3.77)</td>
<td>N (Asparagine)</td>
<td>18 (3.4)</td>
</tr>
<tr>
<td>F (Phenylalanine)</td>
<td>33 (6.23)</td>
<td>Q (Glutamine)</td>
<td>16 (3.02)</td>
</tr>
<tr>
<td>W (Tryptophan)</td>
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<table>
<thead>
<tr>
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<tr>
<td>D (Aspartic acid)</td>
<td>26 (4.91)</td>
<td>K (Lysine)</td>
<td>40 (7.55)</td>
</tr>
<tr>
<td>E (Glutamic acid)</td>
<td>31 (5.85)</td>
<td>R (Arginine)</td>
<td>22 (4.15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H (Histidine)</td>
<td>14 (2.64)</td>
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Modified inversion recovery pulse program to include water pre-saturation:

"d13=3u"
"p2=p1*2"
"d11=30m"
"d12=10u"
  l ze
2 d13
  d12 pl9:f1
  d1 cw:f1
  d13 do:f1
  d12 pl1:f1
  p2 ph1 ; 180 pulse
d13
  d12 pl9:f1
  vd cw:f1 ; variable delay
d13 do:f1
  d12 pl1:f1
  p1 ph2 ; 90 degree pulse
go=2 ph31
d11 wr #0 if #0 zd
d11 ivd
  lo to 1 times l4
exit

ph1=0 2
ph2=0 0 2 2 1 1 3 3
ph31=0 0 2 2 1 1 3 3

; ph1 : f1 channel - power level for pulse (default)
; ph1 : f1 channel - 90 degree high power pulse
; ph2 : f1 channel - 180 degree high power pulse
; d1 : relaxation delay; 1-5 * T1
; d11: delay for disk I/O [30 msec]
; vd : variable delay, taken from vd-list
; l4: l4 = number of experiments = number of delays in vd-list
; NS: 8 * n
; DS: 4
; tdl: number of experiments
; this pulse program produces a ser-file (PARMOD = 2D)
Appendix C

Additional alterations to protein preparation procedure

The following changes were also made to the protein purification in order to try to get the NMR experiments to work. By purifying the protein and then performing a $T_1$ relaxation experiment in the presence of testosterone it was thought that it would be possible to test whether this change had made a difference; since if any large changes in the testosterone proton’s $R_1s$ had been observed this would have been a positive indicator.

<table>
<thead>
<tr>
<th>Altered Step</th>
<th>Change made</th>
<th>Reason</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>I and IV</td>
<td>As in method A1 (Chapter two, section 2.2.1.5), but also added aprotinin (1 μg/mL) and leupeptin (1 μg/mL) as well as PMSF (1 mM). No final HiTrap Q step</td>
<td>To prevent protease activity</td>
<td>No effect on NMR experiments</td>
</tr>
<tr>
<td>II and IV</td>
<td>Tried sonicating only once at 10 microns for 15 seconds No final HiTrap Q step</td>
<td>To reduce sheering forces on P450 3A4</td>
<td>No effect on NMR experiments</td>
</tr>
<tr>
<td>III and IV</td>
<td>20 mM Tris used instead of KPB No final HiTrap Q step</td>
<td>Protein might be more stable in tris buffer</td>
<td>No effect on NMR experiments</td>
</tr>
<tr>
<td>III and IV</td>
<td>Tried reducing E911 concentrations to zero by washing protein on column with buffer containing no detergent No final HiTrap Q step</td>
<td>Protein may be inhibited by the detergent</td>
<td>Reducing E911 had no effect on NMR experiments.</td>
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</tbody>
</table>
### Appendix C

<table>
<thead>
<tr>
<th>Altered Step</th>
<th>Change made</th>
<th>Reason</th>
<th>Effect</th>
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</thead>
<tbody>
<tr>
<td>IV</td>
<td>As in method A1, plus tried using benzamidine in buffer</td>
<td>Protease inhibitor</td>
<td>No effect on NMR experiments</td>
</tr>
<tr>
<td>IV</td>
<td>As in method A1, plus tried using 200 mM KCl in buffer</td>
<td>To prevent aggregation due to sudden change in ionic strength</td>
<td>No effect on NMR experiments</td>
</tr>
<tr>
<td>IV</td>
<td>As in method A1, plus tried using different concentrations of glycerol (20, 15, 10% v/v)</td>
<td>To see if glycerol effects P450 3A4 stability</td>
<td>No effect on NMR experiments</td>
</tr>
<tr>
<td>IV</td>
<td>As in method A1, plus tried different pH buffers 7, 7.5 and 8.</td>
<td>To see if pH effects P450 3A4 stability</td>
<td>No effect on NMR experiments</td>
</tr>
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</table>

In addition, when using method A2 the CHAPS concentration was reduced by washing the HiTrap Chelating column with buffer containing no detergent, since it was thought this detergent could also be effecting the NMR experiments. However, the result of this was less stable protein that precipitated within a day at 4°C.

In retrospect it would have been more informative to use the 7-BQ catalytic assay as a test for whether an improvement in the quality of P450 had been achieved, since the NMR experiments provide only a positive or negative answer.