Electron Transfer Effects in Metalloproteins: An ESR Study

A Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science at the University of Leicester by

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"ELECTRON TRANSFER EFFECTS IN METALLOPROTEINS; AN ESR STUDY"

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ABSTRACT
The metal chromophore in metalloproteins is identified as a high affinity site for dry electrons in conformity with previous studies. Relative electron affinities for such centres in a physiologically probable valence hybrid haemoglobin have been determined. A ratio of ca. 2.5 for the electron affinity of oxyhaemoglobin over methaemoglobin is explained in terms of structural differences in the constituent forms. The phenomenon of intersubunit electron transfer in similar systems is considered in terms of haem edge-to-edge minimum separation.

The hydrogen peroxide complex of iron haemoproteins is characterised as an oxo compound of iron in the +4 oxidation state (ferryl), by electronic spectroscopy and ESR spectroscopy using low temperature γ-irradiation. Variations in ESR parameters during annealing are explained in terms of structural changes at the haem site.

The electron-loss centre in many proteins, consequent upon γ-irradiation, is identified as the polypeptide amide nitrogen. The nitrogen-centred radical first formed may undergo hydrogen transfer reactions to give a terminal carbon-centred radical. ESR features for the nitrogen-centred radical are interpreted in relation to predominant secondary structures in the proteins examined.

The catalytic mechanism of xanthine oxidase has been studied by the use of dry electrons as the reducing substrate. The series of intermediates obtained through annealing have ESR parameters identical with those for the enzyme-substrate intermediates obtained by other workers using chemical reducing substrates. A scheme of intramolecular electron transfer is proposed.
To my devoted Adebimpe
'WHEN THOU GIVEST TO THY SERVANTS TO
ENDEAVOUR ANY GREAT MATTER, GRANT US ALSO
TO KNOW THAT IT IS NOT THE BEGINNING, BUT
THE CONTINUING OF THE SAME, UNTIL IT IS
THOROUGHLY FINISHED, WHICH YIELDETH THE
TRUE GLORY'

Sir Francis Drake

'GOOD SCIENCE AS A WAY OF LIFE IS
SOMETIMES DIFFICULT'

James D. Watson

A F’OPE F’OLORUN

(WE THANK THEE ALL OUR GOD)

A Hymn of Thanksgiving
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STATEMENT

The accompanying thesis submitted for the degree of Ph.D entitled "Electron Transfer Effects in Metalloproteins; An ESR Study" is based on work conducted by the author in the Department of Chemistry at the University of Leicester mainly during the period October 1985 to April 1988. All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other University.

F. Adetokunbo Taiwo
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Chapter 1

Theory of Electron Spin Resonance Spectroscopy
THEORY OF ESR SPECTROSCOPY

1.1 INTRODUCTION

The work presented in this thesis is based mainly on the electron spin resonance (ESR) of metal chromophores in proteins, and associated protein radicals obtained by γ-irradiation or chemical reactivity.

The purpose of this chapter is to make a brief review of the theory of ESR, and such factors which affect vital parameters obtainable from the spectra. Attention is focused on selected metal ions because of their prevalence in the systems studied. Specific references have not been cited for some of the different aspects of this general theory. Interested readers may consult the list of references, some of which are popular texts in the field of ESR study.

1.2 The Electron Spin Resonance Phenomenon

An unpaired electron possesses a magnetic moment by virtue of its spin \( s = 1/2 \). When an external magnetic field is applied, there are two possible orientations of its spin. These are the parallel alignment, \( M_s = +1/2 \), and the antiparallel \( M_s = -1/2 \). Transitions between these two levels can be induced if an oscillating electromagnetic field of appropriate resonant energy is applied perpendicular to the external fixed magnetic field. The resonant energy \( E \) must be equal to the difference in energies between the \( M_s = +1/2 \) and \( M_s = -1/2 \) levels as shown in Fig.1.1.
$\beta$ is the Bohr magneton

$H$ is the applied magnetic field

$h$ is Planck constant

$\nu$ is the frequency of the applied microwave energy

**Figure 1.1**

Effect of an applied magnetic field, $H$, on the $M_s = \pm 1/2$ levels of an unpaired electron, showing (a) the energy splitting, (b) the absorption spectrum, and (c) the first derivative ESR spectrum.
ESR transitions can be viewed as a spin flip in which a precessing electron spin changes its orientation about the axis of an applied magnetic field. At the ground state there is a slightly higher population in the more stable lower energy level. This results in a net absorption of microwave energy which may be detected, amplified, and converted to give the recorded spectrum. Spectra are usually produced as their first derivative curves with respect to the changing external magnetic field.

1.3 The Spin Hamiltonian

The electron in a magnetic field is represented in quantum mechanics by the Schrödinger wave equation

\[ H\psi = E\psi \]

in which \( H \) is the Hamiltonian operator of the wave function \( \psi \), and \( E \) the corresponding eigenvalues, being simple solutions of the equation.

ESR spectroscopy arises from transitions between the eigenstates of the Hamiltonian when the eigenvalues \( E_1 \) and \( E_2 \) are separated by the energy of the applied magnetic field. Unpaired electrons are not isolated free species in space, and are therefore described by the several factors which contribute to their perturbation in real systems. Abragam and Bleaney (1970) have given a detailed mathematical treatment of this theory.

The multivariable spin Hamiltonian of the electron-in-orbit is given by

\[ H = H_{ez} + H_{hf} + H_q + H_{zf} + H_{hz} + H_{shf} \ldots \ldots [1] \]
in which each of the constituent terms contributes to the effective eigenvalues and hence the spectroscopic parameters obtainable for different systems by varying instrument settings.

1.4 Electronic Zeeman Interaction
The interaction between the electronic magnetic moment and an applied magnetic field lifts the degeneracy of the spin states and is represented by the Hamiltonian
\[ H_{\text{ez}} = \beta H (L + 2S) \] ........................[2]
where \( \beta \) is the Bohr magneton, \( L \) and \( S \) are orbital and spin operators respectively. In the absence of orbital momentum \( (L = 0) \), as for a hypothetical free electron,
\[ H = 2 \beta HS = g \beta HS \] ........................[3]
where \( g \) (the g-value) is called the spectroscopic splitting factor.
The g-factor depends on the direction of \( H \), the applied magnetic field with respect to the crystallographic axes \( (x, y, z) \) and thus assumes a 3-dimensional representation;
\[ H = \beta (g_{xx}H_x S_x + g_{yy}H_y S_y + g_{zz}H_z S_z) \] ........................[4]

1.5 Hyperfine Interaction
A magnetic nucleus can interact with the magnetic moment of its associated unpaired electron to produce additional magnetic fields. This results in a multiplet structure of an otherwise single line spectrum, called hyperfine splitting. A nucleus with spin \( I \), when placed in a magnetic field, has \((2I+1)\) orientations. For each electron there are two possible energy levels; giving a total of \( 2(2I+1) \) levels. According to the normal selection rules:
$\Delta m_s = \pm 1$ and $\Delta m_t = 0$, the number of hyperfine lines obtainable is $(2I+1)$. The hyperfine Hamiltonian is given by

$$\mathcal{H}_{hf} = (A_{xx}I_xS_x + A_{yy}I_yS_y + A_{zz}I_zS_z) \ldots \ldots \ldots \ldots \ldots [5]$$

where $'A'$ is the hyperfine coupling constant, which is also normally treated as a second rank tensor.

When the distribution of the unpaired electron is uniform (ie. spherical) about the magnetic nucleus, as in $s$-orbitals, $A_{xx} = A_{yy} = A_{zz}$. The resulting hyperfine coupling is isotropic. In a non-spherical system like $p$- or $d$- orbitals, the hyperfine coupling is anisotropic. Values of $'A'$ therefore contain both the isotropic ($A_{iso}$) and the anisotropic ($B$) components.

1.6 Anisotropic Hyperfine Coupling

If the external magnetic field is aligned parallel to the axis of a $p$-orbital (ie. $z$-axis), the anisotropic component is given the value $2B$. When the field is aligned perpendicular to the $p$-orbital (ie. $x$-$y$ plane), the anisotropic component is now negative and reduced to $-B$. The coupling constants in both cases are given by

$$A_{||} = A_{iso} + 2B \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots [6]$$

and $$A_{\perp} = A_{iso} - B \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots [7]$$

Values of $A_{iso}$ and $B$ cannot be deduced by the direct determination of $A_{||}$ and $A_{\perp}$ from spectra because the signs of the coupling constants are not defined from such experimental data. However $|A_{iso}|$ can be obtained from liquid phase data in which there is a high tumbling rate and quick averaging, giving $g_{av}$ (isotropic hyperfine constants only). Since the relative signs for $A_{||}$ and $A_{\perp}$
are fixed, the isotropic and anisotropic components can be partitioned. The composition of a paramagnetic nucleus in terms of s- and p- character can be obtained from $A_{\text{iso}}$ and $2B$ respectively. Standard values of $A_{\text{iso}}$ and $2B$ (available in Symons, 1978) are compared to experimental data to give approximate values of spin densities in the respective orbitals;

$$a_s^2 = \frac{A_{\text{iso}}}{A_{\text{iso}}}$$ \[8\]

$$a_p^2 = \frac{2B}{2B^o}$$ \[9\]

In the case of d-orbital electrons, there is coupling to the $dz^2$ orbital, identical with that in p-orbitals. For the other d-orbitals, a change in orbital symmetry reverses the sign of the anisotropic interaction, thus giving

$$A_{\|} = A_{\text{iso}} - 2B$$ \[10\]

$$A_{\perp} = A_{\text{iso}} + B$$ \[11\]

1.7 Quadrupole Interaction

This is the interaction between the electric quadrupole moment $Q$ (by effective separation of charge in the nucleus) and the electric field gradient $\delta E/\delta Z$ of its surroundings, set up by both the valence electron and the crystallographic environment, which for $H_0 = 0$ determines the axis of the nuclear spin. This interaction is given by the Hamiltonian:

$$\mathcal{H}_Q = \left\{ 3e_0/[4I(2I+1)] \right\} - \frac{\delta^2 E/\delta Z^2[Iz^2 - \frac{1}{2}I(I+1)]}{\ldots} \[12\]

where $e_0$ is a measure of deviation from spherical distribution of electronic charge.

Quadrupole interaction tries to align the nucleus along
the symmetry axis (electric field axis) while the magnetic
moment due to the electron aligns the nucleus
perpendicular to the same axis. A mixture of nuclear
states thus ensues within which transitions do not obey
formal selection rules. This may result in the appearance
of 'forbidden' transitions; \( \Delta M_I = \pm 1 \), or \( \pm 2 \).

1.8 Zero-field Interaction
In the absence of an external field, the electron and
nuclear spins are coupled, such that the degeneracy of the
ground spin states for the electron tends to be removed.
Thus an energy separation of the \( s = \frac{1}{2} \) states at zero
field is observed as illustrated in Fig.1.3.
The zero-field Hamiltonian is given by
\[
H_{zf} = D[S_z^2 - \frac{3}{2}S(S+1)] + E[S_x^2 - S_y^2]
\]
where \( D \) and \( E \) are zero field splitting constants.
For a nucleus with \( I = \frac{1}{2} \), apparent triplet \((+1, 0, -1)\)
and singlet \((0)\) states are possible though the energy
difference is very small. When an external magnetic field
is applied, the \(|+1/2, -1/2\rangle\) and \(|-1/2, +1/2\rangle\) transitions
are shifted to lower positions on the field axis; the
shift being much greater for the \(|+1/2, -1/2\rangle\) state. This
causes \( A \) and \( g \) values to appear larger than they truly
are. The differential shift becomes more prominent as the
nuclear spin increases as for example in \( \text{Mn(II)} \) complexes.
Such effects of zero-field splitting on spectroscopic
parameters are generally referred to as Breit-Rabi
effects. When \('A' is large the \(|+1/2, -1/2\rangle\) transition
shifts to such a low field that corrections for \('A' and
'B' become important especially on X-band and if \( A \approx B_0 \).
Figure 1.2
Divergence with field of the $M_s = \pm 1/2$ levels when associated with a nucleus having $I = 1/2$, in the high-field approximation. There is no change in $M_I$. 
Figure 1.3
Divergence with field of the $M_s = \pm 1/2$, $I = 1/2$ case in the low-field approximation showing how the zero-field 'singlet' and 'triplet' states arise.
To calculate $A_{iso}$ and $g_{av}$ from field values of the observed transitions, the Breit-Rabi equation (1931) is used.

1.9 Nuclear Zeeman Interaction
The nuclear magnetic moment generated by the nuclear spin may be oriented in $(2I+1)$ ways in an external magnetic field. The Hamiltonian is given by

$$H = g_n \beta_n HI$$

where $g_n$ is the nuclear $g$-factor and $\beta_n$ is the nuclear Bohr magneton. The nuclear Zeeman interaction merely shifts all electronic energy levels to the same extent.

1.10 Superhyperfine Interaction
This arises when the unpaired electron density is delocalised onto surrounding nuclei and therefore experiences additional hyperfine interaction from them:

$$H = SIA^*_i I_i$$

where $A^*_i$ is the superhyperfine interaction tensor exerted by nucleus $i$ of spin $I_i$.

1.11 The Hydrogen Atom
The spectrum of the free electron is a one-line signal at free-spin. In the hydrogen atom, the electron is next to a nucleus with magnetic moment $I = 1/2$. Since there are $2(2I+1)$ energy levels, there are $(2I+1)$ transitions. These are observed as separate lines of equal intensities separated by a hyperfine splitting constant ‘$A$’. During the time in which the electron changes its spin orientation, the nuclear spin remains virtually unaltered.
The energy separation between the two electron-nuclear interactions is depicted in the value of 'A'. Generally the number of hyperfine lines is given by \((2nI+1)\); where \(n\) is the number of equivalent nuclei, and \(I\) the nuclear spin.

1.12 One Nucleus With \(I = 1\)

For \(I = n\), there are \(2(2n+1)\) possible energy levels \((M_s = \pm 1/2)\). By the selection rules; \(\Delta M_I = 0\) and \(\Delta M_s = 1\), there are \(2n+1\) allowed transitions. Therefore for \(^{14}\text{N}\) or \(^2\text{H}\), \(I = 1\), \(s = 1/2\), three lines are obtained as shown in Figure 1.4.

1.13 The g-Value

This is the spectroscopic splitting factor which gives the rate of divergence of the Zeeman splittings for an electron at resonance. It is similar to the Lande factor in atomic spectroscopy, being a ratio of energy and momentum, and hence dimensionless. The most significant of all terms in the multivariable spin Hamiltonian Eqn.[1] is that due to the electronic Zeeman interaction Eqn.[2]. For an isotropic system, \(\mathcal{H}_\text{ez}\) becomes

\[
\mathcal{H}_\text{ez} = \beta g H S
\]

If \(M_s = \pm 1/2\), and \(M_I = 0\),

\[
\Delta E = h\nu = g\beta H_0 \quad \text{(i.e. } 1/2g\beta H_0 - (-1/2g\beta H_0)\text{)}
\]

Often there is an angular variation of \(g\) in terms of direction of \(H\) with respect to the molecular axes of the paramagnetic sample. The \(g\)-values for \(H\) along the three principal molecular axes (\(X\), \(Y\), \(Z\)) are \(g_x\), \(g_y\), \(g_z\). For
Figure 1.4

Energy level diagram for a system with \( s = 1/2 \), \( I = 1 \), showing three lines of equal intensity in the ESR spectrum.
an isotropic system

\[ g_{xx} = g_{yy} = g_{zz}. \]

Two cases of anisotropy often arise. One is when \( H \) is applied along the \( Z \) axis with \( X \) and \( Y \) being not equivalent; rhombic symmetry,

\[ g_{xx} \neq g_{yy} \neq g_{zz}. \]

The other is when \( X \) and \( Y \) are equivalent, leading to two \( g \)-values; \( g_\| \) being along \( Z \) axis and \( g_\perp \) being in the \( XY \) plane. This is axial symmetry,

\[ g_\perp = g_{xx} = g_{yy} \]
\[ g_\| = g_{zz}. \]

Shifts from the free electron \( g \)-value of 2.0023 (based on its spin only momentum), arise from the electron’s orbit around a nucleus. This motion generates an orbital angular momentum which contributes to the net magnetic moment, thus causing a shift in \( g \)-value from free-spin.

1.14 Analysis of Spectra

Ideally the most informative spectra and with the least ambiguity in the interpretation of features are those from single crystal samples. The limitations in the use of single crystals however are inherent in the difficulty of preparation and isolation of the perfect crystal. Further to this, the mounting of a single crystal in the spectrometer cavity involves a particularly delicate technique because of its several possible orientations in space. For the protein molecule the unit crystal can be conveniently described in terms of the rectangular coordinates: \( x \), \( y \), and \( z \), with respect to an endogeneous
Figure 1.5
Idealised ESR spectra for randomly oriented species with g-anisotropy showing the axial and rhombic cases. (A is the absorption spectrum, B is the first derivative spectrum)
metal constituent if present. Otherwise, assignment of axes becomes difficult.

The alternative sample format is the 'disordered' powder sample in the form of a glassy or polycrystalline matrix. The powder spectrum obtained is a statistically weighted envelope of the constituent microscopic elements. The composite spectrum is therefore a cumulative average of all radical features present in the sample. Where more than one radical types are present, multiple features are observed in the emergent spectrum. Generally for the powder spectrum, absorption in the perpendicular direction is twice as intense as in the parallel because twice as many radicals are aligned with their axes perpendicular to the applied field than the parallel. A detailed analysis of powder spectra is given in Atkins and Symons (1967).

When a sample is exposed to ionising radiations, it is often the case that more than one radical is produced. The spectral identification of the different radicals is complicated by overlapping g-values and the predominance of some radicals resulting in the screening of other less intense features. Analysis of such composite spectra is facilitated by the variation of different parameters like microwave power and temperature to saturate out some of the features thus revealing less complex spectra. Different doses of radiation may also produce different yields of radicals. Where there is g- anisotropy, the use of Q-band enhances resolution of insufficiently resolved lines in the X-band spectrum.
The use of on-line computer has also facilitated spectra analysis through the manipulation of spectra recorded at different settings. The simulation and synthesis of spectra for comparison with experimental results is often used to corroborate many interpretations.
REFERENCES


Chapter 2

Haemoglobin Structure and Reactivity
HAEMOGLOBIN STRUCTURE AND REACTIVITY

2.1 INTRODUCTION
A background of information on haemoglobin is presented here for two reasons. First, in parts of this study, the principal protein of interest is haemoglobin. Second, the relationships between structure and function in proteins, as mentioned in other aspects of the work relate to haemoglobin as a model. Haemoglobin is historically one of the most thoroughly researched and cited proteins with central and complex functions in many animals.

2.2 Function
The physiological function of oxygen supply to animal tissues is performed by a group of metalloproteins. In invertebrates, a diversity of molecules are employed for this function, and the main respiratory pigments are chlorocruorins, haemerythrin, erythrocruorin, and hemocyanin. In vertebrates, haemoglobin is the principal oxygen transport medium and myoglobin is used as an oxygen store. Iron is the metal centre in all of these pigments except the copper based hemocyanin. In vertebrates, haemoglobin transports oxygen from the lungs to the tissues, and on its return course, carries carbon dioxide as carboxyhaemoglobin in an acid medium.

2.3 Structure
Haemoglobin is a globular protein comprising four polypeptide chains which are identical in pairs in α- and
\( \beta \)-chains. Each chain is made up of two parts: the proteinoid mass called the globin, and the metallo-organic part called the haem. Each of the four chains is similar to the single myoglobin polypeptide, Fig. 2.1, with very slight but important differences in the primary structure, i.e., identity and sequence of aminoacid residues in the globin.

The globin of the \( \alpha \)-chain is made up of 141 amino acid residues joined by peptide linkages. Correspondingly in the \( \beta \)-chain there are 146 residues, and in myoglobin, 153. The globins are characterised by a number of right-handed alpha helical segments; eight in the \( \beta \)-chain, and seven in the \( \alpha \)-chain. Variation in the primary structure of the globin results in altered haemoglobin phenotypes. Some of these phenotypes possess drastically altered structural and functional characteristics. A most dramatic example is the sickle cell variant, \( HbS \), in which a glutamic acid residue at position 6 on the \( \beta \)-chain is replaced by valine (\( \beta^6 \text{glu}^{-\text{val}} \)). A comprehensive listing of primary structures for several haemoglobins and other proteins has been compiled by Dayhoff and Eck (1968).

The haem comprises an iron atom bonded to the four pyrrole nitrogens of a protoporphyrin IX molecule, Fig. 2.2. Attached to the porphyrin are two propionic acid side chains, two vinyl groups, and four methyl groups. The octahedral stereochemistry of the iron is completed by two bonds. These are on either side of the porphyrin plane, linking the iron with the globin. On one side, the iron is
Figure 2.1
The myoglobin molecule showing the position of the haem, the \(\alpha\)-helix secondary structure, and the tertiary structure.
Figure 2.2
The Protoporphyrin IX Complex in the haem.
covalently bonded to the imidazole nitrogen of histidine F8 residue, the proximal histidine. On the reverse side of the haem plane are the distal histidine E7 and valine E11 between which exists an empty pocket close to the iron. This is the ligand-binding site Fig.2.3.

Figure 2.3
Stereochemistry of the environment of the haem pocket with bound dioxygen.
In oxygenated, or deoxygenated haemoglobin the iron exists in the +2 oxidation state, thus conferring a bright reddish colouration on the protein. When exposed to oxidising agents like the hexacyanoferrate (III), dioxonitrate (III), or copper (II) ions, Fe(II) is converted to Fe(III). This oxidised derivative methaemoglobin, has a characteristic brownish colouration. A concomitant change in the optical spectrum is routinely used for monitoring this oxidation. The relative position of the iron with respect to the porphyrin plane depends on the oxidation state of the iron and the type of ligand present. Low spin Fe(II) is located in-plane. High spin Fe(II) and Fe(III) are slightly out-of-plane. The off-plane distance for high spin Fe(II) is greater than for Fe(III), (Perutz, 1965). All off-plane displacements of iron are towards the proximal histidine.

The four polypeptide chains are arranged tetrahedrally around a two-fold axis of symmetry. In the individual chains there is a non-polar cavity within the folds of the alpha helices. It is here that the heme is located, about 9Å from the surface of the molecule. This hydrophobic cavity has been suggested to be the access route for organic molecules whose reactions with haemoglobin involve the haem (Antonini and Brunori, 1971). The inter-iron distances between like and unlike chains are different. The α-α iron separation is 36Å, the β-β is 33.4Å, and the α-β is 25Å (Perutz et al. 1960; Cullis et al. 1962).
2.4 Ligand Binding Reactions

Deoxyhaemoglobin binds mainly uncharged ligands such as oxygen, carbon monoxide, nitric oxide, and the alkyl isocyanides. Cyanide may also bind, forming a cyano-derivative. Methaemoglobin, with an extra positive charge, binds negatively charged ligands like cyanide, fluoride, azide, cyanate, thiocyanate, formate, and acetate ions. It also binds ligands in which lone pairs of electrons are present, eg. water, nitric oxide, imidazole, ammonia, and the alkylamines. Oxyhaemoglobin being the physiologically essential derivative, makes oxygen the most widely studied ligand, and it is in relation to oxygen binding parameters that most other ligand binding reactions are interpreted and understood.

The oxygenation reaction of haemoglobin was first represented by the equilibrium reaction:

\[ \text{Hb} + 4\text{O}_2 = \text{Hb}(\text{O}_2)_4 \]

with the equilibrium constant

\[ K = \frac{[\text{Hb}(\text{O}_2)_4]}{[\text{Hb}][\text{O}_2]^4} \]

Bohr and coworkers (1904) found that the oxygenation reaction conformed with a sigmoid saturation curve, Fig.2.4. For this, Hill (1910) proposed the aggregation theory:

\[ (\text{Hb})_n + n\text{O}_2 = (\text{HbO}_2)_n \]

from which

\[ K(P_{O2})^n = \frac{[(\text{HbO}_2)_n]}{[\text{Hb}_n]} \]

and \( \log K + n\log(P_{O2}) = \log[y/(1-y)] \)

where \( y = \frac{[(\text{HbO}_2)_n]}{[(\text{HbO}_2)_n] + [(\text{Hb})_n]} \)

A plot of \( \log[y/(1-y)] \) versus \( \log P_{O2} \), known as the Hill plot, is linear, Fig.2.6. The value of 'n' is an index of
Figure 2.4

Oxygen saturation curves for myoglobin and haemoglobin showing hyperbolic and sigmoid patterns for the respective proteins, and their relative affinities for oxygen.
Figure 2.5

Logarithmic plot of fractional oxygen saturation of haemoglobin.
Figure 2.6
The Hill Plot.

Y is fractional saturation of haemoglobin

$P_{O_2}$ is the partial pressure of oxygen
cooperativity, a sequential enhancement of reactivity of the subunits for successive ligand binding. Hill's aggregation theory however breaks down considering the fact that the haemoglobin tetramer is intact all through the reaction and does not polymerise. Secondly the initial and final portions of the sigmoid oxygen equilibrium curve do not conform with Hill-plot linearity. The value of 'n' is therefore best estimated close to y = 0.5.

2.5 Allosteric Transitions

When a ligand binds to haemoglobin there are changes in the conformation of the microenvironment of the binding site. These in effect generate a total change in the gross conformation of the molecule. The parameters of subsequent ligand binding to a slightly different conformation, are therefore different from those of the preceding similar reaction. Such conformational changes are known as allosteric transitions.

Adair (1925) proposed a scheme of successive equilibria in which a step-wise addition of oxygen is used:

\[ \text{Hb}_4 \text{(O}_2\text{)}_{i+1} + \text{O}_2 = \text{Hb}(\text{O}_2)_i; \quad i = 1, 2, 3, 4 \]

and \( y = \frac{\Sigma i K_i P_i}{1 + \Sigma K_i P_i} \)

where \( K_i \) is the equilibrium constant for the \( i \)th equilibrium in the series of four. The fractional saturation \( y \), is therefore described by a four-constant expression in consonance with the four binding sites, each with a slightly different binding constant, or ligand affinity.
Pauling (1935) modified Adair's scheme by introducing pairwise heme-heme interaction in a square model of the tetramer. The fractional saturation \( y \), is given by

\[
y = \frac{K'P + (2\alpha + 1)(K'P)^2 + 3\alpha^2(K'P)^3 + \alpha^4(K'P)^4}{(1 + 4K'P) + (4\alpha + 2)(K'P)^2 + 4\alpha^2(K'P)^3 + \alpha^4(K'P)^4}
\]

where \( K' \) is the intrinsic equilibrium constant for the hypothetical reaction of oxygen with each isolated heme, and \( \alpha \) is the interaction constant between heme pairs. Though this theory eliminates Adair's four constants, \( K_i \) being invariant as would have been the case for independent binding sites where \( n = 1 \) (as for myoglobin), it does not explain why the value of \( n \) for haemoglobin is less than 4, the number of binding sites.

On the basis of differences between hemes, Wyman (1948) created a distinction between interactions of identical and non-identical hemes. The original square model was thus changed to a rectangular one in which \( \alpha \) was replaced by \( \alpha \) and \( \beta \) the respective interaction constants between pairs of \( \alpha \)-chains and \( \beta \)-chains.

Wyman and Allen (1951) later introduced the concept of conformational effects in ligand-binding. Significant differences in conformations of haemoglobin chains and derivatives are reflected in the characteristics of their ligand-binding reactions (Antonini and Brunori, 1971). The effects of conformational differences in haemoglobin structure-function relationships are similar to those often observed in many other proteins (Gerhart and Pardee, 29-
Umberger, 1964; Dickerson and Timkovich, 1975). This makes haemoglobin a model for general regulatory phenomena in biological systems.

In order to understand the mechanism of conformational changes accompanying oxygen-binding, Monod et al. (1965) proposed the 'Concerted Transition Model'. The Monod-Wyman-Changeux (MWC) model assumes that

(i) the protein is composed of identical binding sites which occupy equivalent positions
(ii) the protein is capable of existing in at least two different conformational states
(iii) the different conformational states have different affinities for the ligand
(iv) all conformational states must be in equilibrium with each other.

It therefore asserts that starting with an equilibrium of conformational states, the introduction and consequent binding of a ligand imposes a shift in the position of equilibrium. This shift is caused by allosteric transitions between the conformational states.

Application of the MWC model to haemoglobin implies that deoxyhemoglobin must exist in two states which are usually described as T (tense) and R (relaxed). Their respective oxy-derivatives would then be To and Ro. Perutz (1970, 1979) also supported this theory by pointing out that extensive structural change is triggered by oxygen-binding. The transition from a five-coordinate deoxy- to a six-coordinate oxy-state is also accompanied
by a spin-state transition of Fe(II) from high spin \((S = 2)\) to low spin \((S = 0)\). The four binding sites are assumed to be independent and the binding constants \(K_T\) and \(K_R\) must be different. The thermodynamic free energy of cooperation between the binding sites is estimated by

\[
\Delta G^\prime = -RT \ln \left( \frac{K_R}{K_T} \right)
\]

For the allosteric equilibrium, a free energy of transition between states in liganded or unliganded form is given by

\[
\Delta G = -RT \ln L
\]

where \(L\) is the allosteric equilibrium constant for the \(T \rightarrow R\) transition.

The demand for two conformational states for each derivative, independence of binding sites, and symmetry constraints by the MWC model did not conform with known characteristics of haemoglobin. An alternative model was later formulated specifically for haemoglobin by Koshland and coworkers (1966), known as the 'Sequential Transition Model'.

The Koshland-Nemethy-Filmer (KNF) model assumes that the binding of a ligand to a subunit in a multisubunit protein first induces a local perturbation which changes the conformation of the affected subunit. In effect the gross conformation of the protein changes and this also changes the ligand-binding affinity of the next available subunit. This sequence of events continues until all subunits have been ligated to give a final product whose conformation is different from that of the original molecule or any of the
intermediate species. In this model a number of interaction constants are discernible. There is a ligand-binding constant $K$, which represents the intrinsic affinity for the ligand by an 'isolated' subunit. Consequent upon initial ligand-binding there is an equilibrium in conformational transition due to allosteric transitions and this is described by $K_i$, the allosteric transition constant. The resulting interaction constants are of two types: $K_{ab}$ and $K_{bb}$, where $A$ and $B$ refer to the ligated and unligated derivatives respectively. Combinations of such values of $K_i$, $K_i$, $K_{ab}$, $K_{bb}$, based on geometrical models account for cases of cooperative or non-cooperative ligand-binding by multisubunit proteins. A detailed discussion of structural changes on ligand binding has been done by Baldwin and Chotia (1979).
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Chapter 3

Electron Spin Resonance Spectra

of Haemoglobin
3.1 Spin States of Iron in Haemoglobin

In oxyhaemoglobin (HbO₂), iron is in the +2 oxidation state. The six d-electrons are distributed into the five d-orbitals in such a way that the ligand field splitting energy is greater than spin-pairing energy. This gives a low spin state with S = 0, which makes the HbO₂ molecule diamagnetic. In deoxyhaemoglobin, Hb, a high spin state, S=2 obtains. Though there are four unpaired electrons, the energy transition for the theoretically possible ESR transition is so great that it cannot be traversed by operative microwaves. Deoxyhaemoglobin is therefore not ESR responsive.

In order to study haemoglobin by ESR techniques it has been bound to paramagnetic ligands like O₂⁻ (Symons, 1975), and nitric oxide (Shiga, 1969; Kappl et al. 1985). Iron(II) has also been replaced by cobalt(II), d⁷, S=1/2 (Hoffman and Petering, 1970). Alternatively the iron centre has been oxidised to Fe(III), d⁵, as in methaemoglobin, though this derivative is not representative of the physiologically functional molecule. The indirect study of haemoglobin has also been done by the synthesis of 'picket fence' model porphyrins (Collman et al. 1975).

3.2 The FeO₂ Complex

The metal-oxygen complex formed by the binding of oxygen
to haemoglobin has been mainly represented by two models; an angular end-on structure by Pauling and Coryell (1936);

\[
\begin{array}{c}
\text{N} \\
\text{N} \\
\text{Fe} \\
\text{N} \\
\text{N} \\
\end{array}
\]

and a diradical ionic structure by Weiss (1964);

\[
\text{Fe(III)}\text{O}_2^-
\]

The Pauling model is mainly supported by the theoretical calculations of Dedieu et al. (1976). The Weiss model is supported by Mossbauer spectroscopy (Lang and Marshall, 1966), infra-red spectroscopy (Barlow et al. 1973), and polar solvent effects (Basolo et al. 1975).

### 3.3 Irradiated Haemoglobins

Symons and coworkers (1978a, 1978b, 1978c) in their work on irradiated haemoglobins at 77 K, identified the FeO\textsuperscript{2+} complex as the main electron adduct centre in oxyhaemoglobins. They found two primary species which were characterised by their g tensors and called α and β centres. These were said to have arisen from the two types of polypeptide units constituting the whole molecule. Annealing above 77 K revealed a multiplicity of new structural variants: γ, δ and δ' which emerged from the two α and β centres. All these species decayed at high temperatures (ca. 180 K) to give one composite Fe(III) (g=6) derivative. The difference between α and β centres as shown by their g tensor components was attributed to differential hydrogen-bonding of FeO\textsuperscript{2+} to the distal imidazolium ion or a water molecule within the porphyrin.
environment. An alteration of the porphyrin environment on the distal front should result in new primary centres different from those of normal haemoglobin.

Comparative studies of normal human haemoglobin, HbA, and the worm polychaete annelid, *Glycera dibranchiata* haemoglobin, HbG, in which the distal histidine in the β-chain is replaced by a leucine (Bartlett and Symons 1983), showed a different FeO₂⁻ β centre in HbG. This new electron adduct centre did not anneal to Fe(III). The lower g values obtained for HbG were attributed to a higher spin distribution onto the oxygen ligand as a result of decreased or lack of hydrogen bonding to the FeO₂ complex. The following scheme was therefore proposed:

\[
\begin{align*}
\text{FeO}_2^– \text{-} \text{HA} + e^- & \rightarrow \text{FeO}_2^- \text{-} \text{HA} \\
\text{FeO}_2^- \text{-} \text{HA} & \rightarrow \text{FeO}_2^\text{HA} + A^- \\
\text{FeO}_2^\text{HA} & \rightarrow \text{Fe(III)} \text{-} \text{HO}^- \\
\text{Fe(III)} \text{-} \text{HO}^- & \rightarrow \text{Fe(III)} + \text{H}_2\text{O}_2
\end{align*}
\]

HA represents the imidazole side chain of distal histidine as shown in the structure below.
For haemoglobin Glycera the above scheme terminates at the formation of FeO$_2$---HA, where HA is HOH.

The above scheme is in accord with the results of Phillips and Schoenborn (1981) which show the presence of hydrogen-bonding prior to electron addition. Furthermore it was suggested (Bartlett and Symons, 1983) that proton transfer is more feasible for the FeO$_2$---H$_2$N complex in normal haemoglobin than the FeO$_2$---HOH in Glycera haemoglobin. This must be due to the higher proton donating power of an imidazolium group than water in the non-protic environment of two methyl groups. Comparison with CoO$_2$ centres (Chien and Dickinson, 1972; Addison and Burman, 1983) in which there is greater electron transfer onto the metal centre shows a close resemblance to the Glycera haemoglobin. Vaska (1976) and Basolo and others (1975) had earlier established that charge transfer toward oxygen in the FeO$_2$ complex is facilitated by the presence of the polar group histidine. More recent results by Addison and Burman (1985) on Glycera hemoglobin show that the substitution of distal histidine by the non-polar leucine residue destabilises the aquomet state of normal haemoglobin by sufficient energy (approx. 10 KJ) to imply hydrogen-bonding interaction between distal histidine and coordinated water.

3.4 Variability of $\beta/\alpha$ ratio
The quarternary structure of normal human haemoglobin is a symmetric molecule comprising four polypeptide chains
which are identical in pairs αα, and ββ (Itano and Singer, 1954; Kishner and Tanford, 1964). Electron addition to the FeO₂ units is expected to produce equal populations of FeO²⁻ α and β centres. The results obtained by Bartlett and Symons (1983) show a departure of β/α ratio from unity. It is greater than 1 and depends on both pH and concentration of inositol hexaphosphate, IHP. This observation suggests an inequivalence in the microenvironment of the FeO₂ in the two polypeptide chains. The fact that α and β centres are distinguishable by differences in their g values also corroborates this inequivalence.

Differences in the intrinsic properties of the constituent chains were first reported by Brunori et al. (1967). This was later supported by kinetic (Gibson et al. 1969), thermodynamic (Antonini et al. 1964), and ¹H-NMR (Davis et al. 1969) measurements. Banerjee and Cassoly (1969) determined redox potentials of isolated haemoglobin chains and found that the redox potential of Fe(II) in the β chain is 60mV more positive than that in the α chain. It follows that the β Fe(II) is more prone to oxidation than its α analogue. In the Cu(II) induced oxidation of haemoglobin (Antholine et al. 1984), the β chain has been identified as a selective oxidation site in preference to the α. It is thus evident that the β/α ratio should be greater than 1.

The structural origin of Bohr effect and allostery in haemoglobin is founded in heterotropic interactions.
between the haem environment and the binding sites of allostERIC effectORS like IHP and DPG. The intermediary events between these two sites are effected by the so called haem-linked ionizable groups (Antononi and Brunori, 1971). Extensive X-ray studies by Garby and coworkers (1969), Brun and Briehl (1970); and reactivity studies by Muirhead (1967); and, Renthal and Benesch (1970), have shown that organic phosphates bind to haemoglobin at a site close to val NA(1β), his H21(143β), and lys EF6(82β). Two of these positions:— val NA(1β) and his H21(143β) have also been identified for Cu(II) binding to haemoglobin as a precursor to Fe(II) oxidation. The his H21(143β) appears to be very crucial in facilitating the oxidation of β Fe(II) through a series of direct salt linkage effects between the imidazolium anion and FeO2 units, or indirect hydrogen-bonding effects between them via water molecules in the protein interior.
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-41-
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Chapter 4

Electron-Capture and Electron-Transfer in Valence Hybrid Haemoglobins
4.1.1 INTRODUCTION
The mechanism of electron transfer reactions in different proteins, particularly those involving enzymatic activities, have been investigated from the basis of oxidation-reduction processes. A large number of such proteins contain metal ions which are buried in the protein interior, a region of low dielectric constant. Inspite of the large separations between metals in different molecules, and also metal to solvent separation, the vital redox reactions progress at reasonably rapid rates. These reactions depend essentially on the phenomenon of electron transfer over long distances both in fluid media and the rigid cryogenic matrices in which particle dynamics is highly constrained.

4.1.2 Electron Capture Events
Considerable attention has been given to metalloproteins because of their prevalence as well as widespread participation in biological redox reactions. In some of the early radiolytic work on proteins by Symons (1975), he irradiated whole blood suspension with γ-rays from a 60Co γ-ray source. In the extensive ESR work that followed, Symons and coworkers used isolated haemoglobins (Symons and Petersen, 1978a, 1978b, Bartlett and Symons, 1983). Their results include the identification of the metal ion in the haem as the primary electron-gain centre.
Results obtained using hemocyanin (Symons and Petersen, 1978c), and Cu/Zn superoxide dismutase (Symons and Stephensen, 1983) confirm that the metal chromophore is the most electron affinic site. Several other studies of low temperature irradiated proteins support the concept that electrons generated in proteins during radiolysis do not necessarily react with the protein in the immediate vicinity of their ejection. They migrate long distances to electron-affinic groups. The host of such groups other than the metal centre are: the R-S-S-R units (Rao et al. 1982), tryptophanyl side groups (Liming and Gordy, 1968), and tyrosyl side groups (Sevilla et al. 1977).

4.1.3 Inter- and Intra-molecular Electron Transfer

In the use of X-rays or γ-rays for ejection of electrons, and their subsequent capture, electron transfer can be described as intramolecular. This is because the distance traversed is at maximum the separation of the metal chromophore from the periphery of the protein molecule. This distance is in fact the nearest approach of solvent molecules. Most of the above mentioned works fall into this category. Intermolecular electron transfer would require the presence of at least two protein units between which the electron could travel.

Winkler (1982), and Isied (1982), with their respective coworkers modified cytochrome c by binding the redox-active pentaammino-ruthenium (III) to a specific histidyl imidazolate side group (His 33). This provided a near-surface site from which electrons could travel to the
native Fe(III) in either the same or an adjacent molecule. A photoexitation of the Ru(III) and subsequent reduction of the Fe(III) in the cytochrome c molecules provided data (Nocera et al. 1984) for comparison of intermolecular and intramolecular activation energies. Electron transfer rates over fixed crystallographically determined distances were also compared.

Studies of intermolecular electron transfer have been made possible through the use of semi-synthetic systems in which the interacting protein units bear different metal ions. Simolo and coworkers (1984), and McLendon and Miller (1985) studied the temperature profile of electron transfer between the iron-porphyrin centres in cytochrome c/cytochrome b5 complex (McLendon et al. 1985) and in cytochrome c peroxidase/cytochrome c complex (Cheung et al. 1986). They concluded that the electron moves through distances in the ranges 8Å to 16Å, and 16Å to 24Å respectively, and with the respective rate constants of 1.6 x 10⁻¹ s⁻¹ and 1 s⁻¹. One of the important conclusions drawn from their work is that the reorganization energy for the proteins following electron transfer is large, being in the range 1 to 2 V.

In a separate study, Peterson-Kennedy et al. (1984, 1986) used mixed metal haemoglobin hybrids in which the α- and β- chains were differently reconstituted to give α₂(Zn)β₂(Fe) and α₂(Fe)β₂(Zn) tetramer species. They measured electron transfer in the Zn(II)/Fe(III) haemoglobin hybrids. The redox-reactive system was induced
by flash photolysis to produce an excited $^{3}\text{Zn(II)}\text{P}$ centre. This acts as an electron donor as shown below:

$$^{3}\text{Zn(II)}\text{P} + \text{Fe(III)P} \rightarrow \text{Zn(III)P}^{*} + \text{Fe(II)P}; \Delta E = 0.8\text{V}..[1]$$

The Zn(III)P* is a π-radical cation which is unstable and undergoes spontaneous decay by electron return:

$$\text{Zn(III)P}^{*} + \text{Fe(II)P} \rightarrow \text{Zn(II)P} + \text{Fe(III)P}; \Delta E = 1.0\text{V} ....[2]$$

From the standard electromotive force of the two processes, reaction [2] is considerably faster than [1]. Considering that the separation between metal centres in α-β hemes is ca. 25Å (Cullis et al. 1962) these reactions must be fast. Their rate constants are of the order of 55 s\(^{-1}\) at ca. 293 K, levelling off in the 150 to 77 K range at 8±2 s\(^{-1}\).

One very important observation in the mixed metal hybrid haemoglobinbs is that the outer electron in the photoexcited triplet state of the zinc-porphyrin unit is probably located not in the zinc orbital but in the diffuse π-electron system of the porphyrin ring. In such a situation the distance travelled by the electron between two heme units would be the edge-to-edge separation of two porphyrins in an α-β couple which in this case is ca. 19Å. In a different investigation on the oxidation of ferro proteins by peroxidases, Poulos and Kraut (1980) had observed that the electron transfer between two protein units is a direct effect between their porphyrin systems
at the distance of closest approach. The haem edge-to-edge distance in their system was 16Å. A generalisation for long distance electron transfer between haemoproteins was therefore made on the basis of direct edge-to-edge haem separation.

In this study, the valence hybrid mixture containing methaemoglobin (Fe(III)) and oxyhaemoglobin (Fe(II)) has been used for two main reasons. On the one hand this system represents a physiologically probable state of methaemoglobinæmia, a condition in which the methaemoglobin reductase enzyme is deficient, thus allowing high amounts of methaemoglobin to remain in equilibrium with the functional oxyhaemoglobin. On the other hand haemoglobin and its derivatives have been so well characterised that mixed reactivities are explicable in terms of structural differences.
EXPERIMENTAL

4.2.1 Preparation of Haemoglobin

Haemoglobin was extracted from fresh human blood using the method of Drabkin (1949). It was stripped of 2,3-diphosphoglyceric acid (DPG) by passage through a Dintzis column (1952). Concentrations of haemoglobin solutions were routinely determined by measuring the optical density of the methaemoglobin cyanide at 540nm using an extinction coefficient of $4.6 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$ according to Drabkin (1949).

4.2.2 Haemoglobin Hybrids

Mixed oxidation states comprising met- and oxy-haemoglobin [Fe(III)/Fe($O_2$)] hybrids were prepared by adding suitable concentrations of ethylene glycol to unbuffered aqueous haemoglobin solutions. The final proportions of the two forms were estimated by measuring the visible spectra of the solutions with a Perkin Elmer 340 spectrophotometer. In an alternative preparation, samples of buffered haemoglobin solutions were partially oxidised to the met- state using varying amounts of tripotassium hexacyanoferrate(III). The resulting tetrapotassium hexacyanoferrate(II), and residual amounts of the oxidising agent were removed by dialysis. Final compositions of solutions containing a range of Fe(III)/FeO$_2$ mixtures were determined using optical densities and the simultaneous equations of Benesch and coworkers (1973). The concentration of deoxyhaemoglobin

-48-
was invariably low in these preparations, ca. 5\%.

When Fe(III)/Fe(II) mixtures were required, samples were deoxygenated by prolonged flushing with genuine oxygen-free nitrogen. Traces of oxygen found present in bottled nitrogen (from BOC Gases) were removed by bubbling through a battery of chromium(II) chloride solution (chromium(III) chloride reduced by nascent hydrogen \textit{in-situ}). Progressive loss of oxygen in haemoglobin solutions was monitored by using a Clark-type Rank oxygen electrode.

4.2.3 Sample Irradiation
Small columns of solutions were frozen to 77 K in glass tubings. The pellets formed were ejected into liquid nitrogen for irradiation. Samples were generally exposed to $^{60}\text{Co} \gamma$-rays in a Vickrad chamber at a dose rate of ca. 0.6 Mrad/h. for up to 2h.

4.2.4 ESR Spectra
X-band ESR spectra were recorded on a Varian E109 spectrometer at 77 K. Samples were annealed either using a Varian V6040 variable temperature system, or by decanting the coolant from the insert Dewar, and allowing the samples to warm to suitable temperatures, while watching rapid spectral changes, and recooling to 77 K for measurement. Relative peak intensities were standardised using a tris(acetyl acetonato) ferrate(III) standard. All g-values were calculated using a Bruker B-H12E field calibrant.
RESULTS AND DISCUSSION

4.3.1 Partial Oxidation of Oxyhaemoglobin
Addition of ethylene glycol to unbuffered solutions of oxyhaemoglobin causes oxidation of the iron from +2 to +3 oxidation state. This is evident from the spectrum of oxyhaemoglobin changing to that of methaemoglobin (Fig. 4.1). The peaks at 541nm and 577nm are simultaneously lost as the one at 630nm grows in. This was again confirmed by the observed increase in the concentration of high spin Fe(III), using ESR measurements. The g=6 signal increased as the methaemoglobin concentration increased.

4.3.2 Effect of Ethylene Glycol
Plots of percentage oxyhaemoglobin and methaemoglobin against ethylene glycol (Fig. 4.2) each shows a sigmoid variation which is characteristic of ligand binding to haemoglobin. This also indicates that the binding is cooperative though the extent of cooperativity has not been determined. That the binding is cooperative is enough evidence that more than one molecule of ethylene glycol reacts with the haemoglobin tetramer. The reactivity at one site affects positively the reactivity at the next site (as explained in Chapter 2).
Figure 4.1
Changes in the electronic spectrum of oxyhaemoglobin (i) due to its oxidation to methaemoglobin (vi).
Figure 4.2
Variation in the concentrations of oxyhaemoglobin ●, methaemoglobin ■, and deoxyhaemoglobin ▲, with ethylene glycol.
4.3.3 Electron Addition to Valence Hybrid Haemoglobin.

The two major reactions expected upon irradiation of Fe(III)/Fe(II) haemoglobin hybrid are:

\[
\begin{align*}
\text{Fe(III)} + e^- & \rightarrow \text{Fe(II)} \quad \ldots \ldots \ldots [1] \\
\text{FeO}_2^+ + e^- & \rightarrow \text{FeO}_2^- \quad \ldots \ldots \ldots [2]
\end{align*}
\]

The \(\alpha\)- and \(\beta\)- species of \(\text{FeO}_2^-\) arising from reaction [2] are shown in Fig.4.3. On annealing, the following reactions are expected:

\[
\begin{align*}
\text{FeO}_2^- + \text{Fe(III)}_{\text{residual}} & \rightarrow \text{FeO}_2 + \text{Fe(II)} \ldots \ldots \ldots [3] \\
\text{FeO}_2^- + \text{2H}^+ & \rightarrow \text{Fe(III)} + \text{H}_2\text{O}_2 \ldots \ldots \ldots \ldots [4]
\end{align*}
\]

In reaction [3] an intermolecular electron transfer would give either (a) a simultaneous loss of both \(\text{FeO}_2^-\) and \(\text{Fe(III)}\) species, or (b) a simultaneous gain of both \(\text{Fe(III)}\) and \(\text{FeO}_2^-\) species. The latter alternative is ruled out because \(\text{FeO}_2^-\) species are not observed in unirradiated haemoglobin, even after autoxidation may have taken place.

In the examination of the forward reaction [3], the post-irradiation products of the hybrid mixture was stored at 77K for an infinitely long period (of nine weeks). No change in the population of either of the two species was observed.

Earlier work by Symons and Bartlett (1983) had proposed reaction [4]. It is here proposed that the overall reaction during the gradual annealing of the system, is a combination of reactions [1], [2], and [4]. A schematic variation of the \([\text{Fe(III)}], \; g = 6\) signal with temperature is given in Fig.4.4.
Figure 4.3
First derivative X-band spectrum for γ-irradiated oxyhaemoglobin at 77 K showing features assigned to the α- and β- FeO₄⁻ species.
Figure 4.4
Schematic diagram showing initial loss (B) of the Fe(III) g=6 signal, followed by gain (A) of the same feature on annealing.
'B' represents the loss in Fe(III) according to reaction [1].

'A' represents the gain in Fe(III) according to reaction [4] after reaction [2]

\[
\begin{align*}
B & \propto [\text{Fe(III)}] \\
A & \propto [\text{FeO}_2] \\
B/[\text{Fe(III)}] & = K_1 \\
A/[\text{FeO}_2] & = K_2 \\
A/B & = [\text{Fe(II)}]/[\text{Fe(III)}] \times K_2/K_1
\end{align*}
\]

A plot of A/B against [FeO2]/[Fe(III)] gives a slope of \(K_2/K_1\) and intercept 0, Fig.4.5.

In the situation that \(K_1 = K_2\), the two reactions [1] and [2] would be equally likely events. This is therefore similar to a case of equal potential energy wells in an energy profile for the reaction. The results give a ratio \(K_2/K_1 = 2.5\). This implies that the two reactions are energetically different and can be linked with to different potential energy wells. Electron capture by FeO\(_2\) is a more likely event by the order of at least 2.5 over reduction of Fe(III) to Fe(II).

4.3.4 Intermolecular Electron Transfer

In view of the very small temperature coefficients found by Petersen-Kennedy et al. (1984,1986), and the fact that the FeO\(^-\) centres react thermally to give H\(_2\)O\(_2\), the detection of reaction [3] at 77 K was not necessary.
Figure 4.5
Correlation between the relative concentrations of Fe(III) and FeO$_2$ units with the A/B ratio.
It may be accepted that the distribution of FeO$_2^-$ and Fe(III) centres in each haemoglobin tetramer need not reflect the overall distribution of these centres, because of the cooperativity of oxygen binding. However any such bias should have little effect on reaction [3] unless all four sites are occupied by oxygen. In this event, electron capture will be statistical, and reaction [3] cannot occur. The results, which give a constant relative preference for addition to the FeO$_2$ units than to the Fe(III) by a factor of ca. 2.5, clearly eliminate this possibility.

It is evident that addition of electrons to FeO$_2$ units is a lot more favourable than to Fe(III) centres. This in turn implies appreciable energy barriers for electron addition, which must be greater for the Fe(III) than the FeO$_2$ units. The explanation of this is that for the high-spin Fe(III) unit there should be a marked coulombic preference, but electron addition into the antibonding 3d($\pi$) orbital(s) requires considerable movement of ligands, and possibly, loss of the sixth ligand. However, addition to the FeO$_2$ unit may involve the lengthening of the Fe-O and the O-O bonds, as well as other possible conformational changes in the protein. The conclusions at this stage are therefore confined to the establishment of a kinetic preference of the FeO$_2$ units for excess electrons.

This establishment of the presence of energy barriers fits
in with the observation that at least under certain conditions, addition to FeO\(_2\) in \(\beta\)-chains is favoured over that to FeO\(_2\) in \(\alpha\)-chains (Symons and Petersen 1978). Structural differences for the parent units must be small but are clearly kinetically significant. This is supported by the studies of isolated haemoglobin chains which showed that the Fe(III)/Fe(II) potential for \(\beta\)-chains is 60mV more positive than that for the \(\alpha\)-chains (Banerjee and Cassoly 1969).

The present inability to detect reaction [3] is in marked contrast with the relative rapid reactions observed for the \(^{3}\text{Zn}\) unit studied by Petersen and others (1984,1986). Had their rate constants been similar to those in this work, reaction [3] would have been almost complete by the time the ESR spectra were obtained, so no FeO\(_2^-\) centres would have been detected. Two factors contribute to this difference. One is that the energy difference in this case is probably much less than the large difference (0.8V) for the \(^{3}\text{Zn}-\text{Fe}\) units. The second factor is that the orbitals involved in the present study are strongly confined to the iron, or iron-oxygen regions, the crystallographic separation being ca. 25 \(\AA\) for the \(\alpha-b\) units. However in the \(^{3}\text{Zn}\) case, the mobile electron is probably on the periphery of the porphyrin, being a more favourable state for porphyrin-porphyrin transfer.

This type of electron transfer mechanism is in line with deductions by Ferguson-Miller (1978) and Osheroff (1980) and their coworkers, on the exposure of the heme edge in
cytochrome c during reactivity. Electron transfer in this case is understood to take place across edge-to-edge distances between the reductase enzyme species and cytochrome c at one end, and between cytochrome c and the oxidase enzyme species at the other end.
CONCLUSION

Irradiation of oxyhaemoglobin by $\gamma$-rays from a $^{60}$Co source results in electron capture at the FeO$_2^-$ units to give the FeO$_2^-$ species at 77 K. In methaemoglobin the electron capture site is the Fe(III) centre which gets reduced to Fe(II).

In a hybrid mixture of haemoglobin derivatives comprising the valence states Fe(II) (as in oxyhaemoglobin), and Fe(III) (as in methaemoglobin), electron-capture occurs at both the FeO$_2^-$ and the Fe(III) centres. FeO$_2^-$ is the more electron affinic site, with an efficiency of ca. 2.5 over that of Fe(III).

Upon storage of the electron adduct FeO$_2^-$ with residual Fe(III) at 77 K there was no detectable intersite electron transfer between FeO$_2^-$ and Fe(III). Were there to be any such phenomenon within the same molecule or between different molecules, a loss of both species would have been observed after prolonged incubation.
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Chapter 5

The Ferryl Derivative
THE FERRYL DERIVATIVE

5.1.1 INTRODUCTION

The occurrence of iron in the +4 oxidation state in haemoproteins was first mentioned by George and Irvine (1952) in the reaction between metmyoglobin and hydrogen peroxide. It was then characterised by optical spectroscopy and given the structure FeO$^{2+}$ in relation to the vanadyl ion VO$^{2+}$. Magnetic susceptibility measurements pointed to the presence of four unpaired electrons suggesting a d$^4$ configuration for Fe(IV).

Since that first investigation the formation of the ferryl derivative as an intermediate has been implicated in several biological redox processes that involve iron. Such reactions include the decomposition of hydrogen peroxide produced in the dismutation of the superoxide radical, O$_2^-$ (Misra and Fridovich, 1971); the autoxidation of ferredoxins (Misra and Fridovich, 1972), and haemoproteins (Cassell and Fridovich, 1975); and the reduction of dioxygen to water by cytochrome c oxidase without the release of O$_2^-$ (Antonini et al. 1970; Witt and Chan, 1987). Leghaemoglobin is also known to react with hydrogen peroxide to give the ferryl complex, (Aviram et al. 1978). Basically, oxidation of Fe(II) to Fe(III) is a single-electron transfer reaction which may progress to the formation of Fe(IV) only in the presence of a sufficiently powerful oxidising agent.
5.1.2 Fe(IV) as an Unstable Intermediate

The formation of Fe(IV) from Fe(II) was hence described as a one step two-electron transfer process lacking an intermediate Fe(III) species (Aviram et al. 1978). It is the decay of the unstable Fe(IV) which finally gave rise to a terminal Fe(III) product. In a recent investigation by Whitburn (1987), oxymyoglobin was reported to react with an excess of hydrogen peroxide to produce an unstable intermediate which decayed to the ferryl derivative. A large excess of hydrogen peroxide reproduced the oxymyoglobin molecule. One interesting aspect of this result is that the two products were in competitive equilibrium. In the same set of reactions, deoxyhaemoglobin was also found to react with hydrogen peroxide, and in fact faster than the oxygenated protein, to give the same products. The results obtained by Puppo and Halliwell (1987), in the reaction of hydrogen peroxide with methaemoglobin and oxyhaemoglobin also point to the formation of the ferryl complex in addition to a free radical suspected to be OH⁻.

In order to detect the unstable intermediate(s) in the reaction of hydrogen peroxide with haemoproteins, Gasyna (1980) performed a 'rapid-freeze' of the reaction mixture followed by an ESR analysis pre- and post- γ-irradiation at 77 K with annealing. The results point to the presence of Fe(IV) pre- irradiation. The Fe(IV) was hence reduced by electron-capture to a low spin Fe(III). It is surprising however that the g-values of the Fe(III) are different from those of normal low spin Fe(III) in
alkaline metmyoglobin. Secondly, the $\Delta g$ for the new low spin Fe(III) is smaller than that of normal low spin Fe(III) in haemoproteins. This new Fe(III) species also decayed on annealing above 77 K, through an intermediate to the high spin Fe(III) ($g = 6$), species. This annealing behaviour is similar to that of the electron adduct in oxyhaemoglobin and oxymyoglobin, the FeO$_2^-$ species, (Symons and Petersen, 1978).

5.1.3 Participation of the Porphyrin Complex in Oxidative Reactions

In a detailed investigation on the redox states of iron in horseradish peroxidase, Yamada and Yamazaki (1974) proposed a scheme of proton dependent equilibria between different derivatives of the iron complex with participation from both the porphyrin and the protein moiety (Figure 5.1). Horseradish peroxidase is a haemoprotein like myoglobin and haemoglobin, but differs in one respect. While the respiratory proteins have their sixth ligand position free for oxygen binding, the corresponding position in horseradish peroxidase is occupied by protein-bound arginine directly coordinated to the iron centre.

Earlier studies on the reaction of horseradish peroxidase with hydrogen peroxide (Ehrenberg, 1962), provided magnetic susceptibility data for the products in order of formation; called compounds I, II, and III. The data suggested the presence of Fe(V) in compound I, and Fe(IV) in compound II. Excess hydrogen peroxide reacted with
Figure 5.1
Proton balance in conversions between redox states of iron in biological oxidative reactions (from Yamada and Yamazaki, 1974)
compound II irreversibly to give compound III. The green colour in compound (II) was attributed to a \( \pi \)-radical cation formed by electron-loss from the porphyrin. This was supported by ESR results in which an intense signal at free spin was a major feature in the spectrum. Dolphin and coworkers (1971) also obtained the green porphyril radical in horseradish peroxidase, chloroperoxidase, and lactoperoxidase. Similar experiments on cytochrome c peroxidase by Yonetani et al. (1966) and Myers and Palmer (1985), produced a radical in the complex 'ES' which was this time attributed to an aminoacid and not the porphyrin because of the absence of the green colour. Gibson and Ingram (1958) had suggested a protein-bound free radical in the reaction between metmyoglobin and hydrogen peroxide. In a similar study by King and Winfield (1963), two such radicals were proposed because of the two peaks observed in the ESR spectrum.

The significance of these results is the fact that at least one free radical is formed, although published consensus on the number or types of radicals does not exist. Another significant difference between the respiratory proteins (myoglobin and haemoglobin) and the oxidoreductase proteins (horseradish peroxidase, catalase, and the cytochrome c redox enzymes) is that the peroxidation is slow in the case of the former and fast for the latter (King and Winfield, 1963).

In view of the fact that most peroxidation reactions involving iron, either in the +2 or +3 oxidation state
give the high spin Fe(III) as a terminal product via the Fe(IV) complex and free radicals, it is important to (i) investigate the formation and structure of the Fe(IV) complex, (ii) determine the participation of the protein moiety in the redox reactions, and (iii) establish any relationships between Fe(IV) and the FeO$^-$ species.

One very important implication of the occurrence of Fe(IV) in biological redox reactions of iron is that it may participate as an unstable intermediate in the conventional Fenton reaction:

(i) Fe(II) + H$_2$O$_2$ $\rightarrow$ Fe(III) + OH$^-$ + OH$^-$

(ii) Fe(III) + O$_2^-$ $\rightarrow$ O$_2$ + Fe(II)

If Fe(IV) is indeed an intermediate, its occurrence would be in reaction [1], this calls to question its manner of formation.
5.2.1 Stock Solutions of Mb., Hb., and Hydrogen Peroxide

Sperm whale myoglobin was obtained from Sigma Chemical Company, usually in the oxidised met-form. To obtain pure metmyoglobin a solution of the myoglobin was oxidised by a two-fold excess of tripotassium hexacyanoferrate(III). The resulting mixture was dialysed overnight against the appropriate buffer. Solutions of oxyhaemoglobin and methaemoglobin were obtained as described in section 4.2.1.

Concentrations of haemoglobin and myoglobin solutions were routinely determined by measuring the optical density of the met-cyanide complex at 540nm. The molar extinction coefficients used were $4.6 \times 10^4 \text{ Mol.}^{-1} \text{ dm}^{-3}$ for haemoglobin tetramer, and $1.15 \times 10^4 \text{ Mol.}^{-1} \text{ dm}^{-3}$ for myoglobin, according as Drabkin (1949).

Hydrogen peroxide solutions were freshly made prior to each experiment. The concentration of each solution was determined titrimetrically against standardized solution of potassium tetraoxomanganate(VII);

$$2\text{MnO}_4^- + 5\text{O}_2^- + 16\text{H}^+ \rightarrow 2\text{Mn}^{2+} + 8\text{H}_2\text{O} + 5\text{O}_2$$

5.2.2 Reaction of Mb. and Hb. with Hydrogen Peroxide

Calculated volumes of hydrogen peroxide were added to each of the protein solutions to give [protein:peroxide] molar ratios of 1:10. After each addition the reaction mixture
was rapidly frozen in liquid nitrogen. Reaction times were estimated as the interval between mixing of reactants and freezing of mixtures.

5.2.3 γ-Radiation and Spectra

γ-Radiation and ESR recordings were performed as described in Sections 4.2.3 and 4.2.4.

Electronic spectra were recorded on the Perkin-Elmer 340 self-recording spectrophotometer at 298 K.
RESULTS

5.3.1 Oxidation of Fe(III) by Hydrogen Peroxide

The reaction between metmyoglobin and hydrogen peroxide was monitored at different reaction times by ESR spectroscopy at different temperatures. The series of time-dependent spectra are shown in Fig.5.2 to 5.4. Addition of hydrogen peroxide to methaemoglobin causes an immediate loss of the g=6, Fe(III) signal. The resulting complex is ESR silent except for a weak signal at free spin. A free radical is thought to have been formed simultaneously with the destruction of Fe(III). The complex formed is unstable in acid medium, pH 6.0 (Fig.5.2). In contrast in alkaline medium, the complex is quite stable, as shown by the non-appearance of the g=6 signal even after a reaction time of 15 min. at pH 9.0 (Fig.5.4). This stability made possible the recording of the electronic spectrum shown in Figure 5.5.

5.3.2 Electronic Spectrum of the Fe(IV) Complex

Optical spectra at pH 9.0 (Fig.5.5) show the difference between methaemoglobin and its complex with hydrogen peroxide. The spectrum of the complex is very similar to that by King and Winfield (1963), both having the same isosbestic point and the same absorption maxima. Comparison with the results obtained by Gasyna (1980) shows that the wavelengths of characteristic peaks are similar but the relative extinction coefficients differ. The difference may not be unconnected with the presence of...
Figure 5.2
ESR spectra of methaemoglobin pH 6.0, plus hydrogen peroxide, frozen to 77 K at different reaction times (i) no $\text{H}_2\text{O}_2$, (ii) 20s. (iii) 2m. (iv) 4m. (v) 15m.
Figure 5.3
ESR spectra of methaemoglobin pH 7.4, plus hydrogen peroxide, frozen to 77 K at different reaction times
(i) no H$_2$O$_2$, (ii) 20s, (iii) 2m, (iv) 4m, (v) 15m.
Figure 5.4
ESR spectra of methaemoglobin pH 9.0, plus hydrogen peroxide, frozen to 77 K at different reaction times
(i) no $\text{H}_2\text{O}_2$, (ii) 20s. (iii) 2m. (iv) 4m. (v) 15m.
Figure 5.5
Optical spectra showing (a) methaemoglobin at pH 9.0, and (b) its conversion to ferryl myoglobin upon addition of hydrogen peroxide.
ethylene glycol in Gasyna's system and that his spectra were recorded at 77 K whereas those in Fig. 5.5 were recorded at 293 K.

The spectrum in Figure 5.5 is identical in the visible region with that obtained by Puppo and Halliwell (1987). A similar spectrum had also been obtained by Bagger and Williams (1971) for horseradish peroxidase compound II. In the soret region the shift of λ_max from 412 nm to 425 nm is identical with that obtained by Yonetani and Schleyer (1966). It is apparent that the common spectrum arises from similar products of hydrogen peroxide reactions with different proteins.

5.3.3 Reduction of Fe(IV) by Gamma Radiation

Exposure of the methaemoglobin-hydrogen peroxide complex to ^60^Co γ-radiation at 77 K results in the emergence of a low spin iron(III) species with g-values (2.43, 2.12, 1.93) herein referred to as 'a', Fig. 5.6. Annealing above 77 K shows a gradual decay of the first-formed 'a' to a series of new low spin iron(III) species herein referred to as 'b', 'c', and 'd'. The series of spectra obtained through an annealing sequence is contained in Fig. 5.7.

Electron spin resonance parameters for iron(III) species obtained in this and other studies are listed in Table 5.1. The g-values of species 'a' obtained in this study compare favourably with those ascribed to the ferryl-derived Fe(III) by Gasyna (1980). It is inferred (see discussion) that the first-formed metmyoglobin-
Figure 5.6
ESR spectra showing the reduced species, low spin Fe(III), from γ-irradiated Fe(IV) complex
Figure 5.7
Annealing sequence spectra of γ-irradiated ferryl myoglobin showing the formation of low spin Fe(III) species 'a' to 'd' and a final high spin Fe(III)
hydrogen peroxide complex is the ferryl derivative, Fe(IV) usually formulated formally as an oxo derivative [Fe(IV)O^2-]. On irradiation Fe(IV) captures one electron and reduces to Fe(III) species 'a', Figure 5.6. On annealing, 'a' relaxes to 'd' through the intermediates 'b' and 'c'. Species 'd' eventually undergoes a spin state transition to the high spin Fe(III) species terminally observed.
TABLE 5.1
ESR parameters for FeO$_2^-$ species

<table>
<thead>
<tr>
<th>Species</th>
<th>$g_1$</th>
<th>$g_2$</th>
<th>$g_3$</th>
<th>Temp./K</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>OxyMb.</td>
<td>2.209</td>
<td>2.111</td>
<td>1.971</td>
<td>77 K</td>
<td>Petersen $^\dagger$</td>
</tr>
<tr>
<td></td>
<td>2.295</td>
<td>2.164</td>
<td>1.942</td>
<td>ca.150 K</td>
<td>Symons (1978)</td>
</tr>
<tr>
<td>OxyHb.</td>
<td>2.240</td>
<td>2.145</td>
<td>1.958</td>
<td>77 K</td>
<td>&quot;</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>2.201</td>
<td>2.113</td>
<td>1.967</td>
<td>77 K</td>
<td>&quot;</td>
</tr>
<tr>
<td>$\delta$</td>
<td>2.217</td>
<td>2.124</td>
<td>1.981</td>
<td>ca.150 K</td>
<td>&quot;</td>
</tr>
<tr>
<td>$\chi$</td>
<td>2.286</td>
<td>2.166</td>
<td>1.945</td>
<td>ca.150 K</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

TABLE 5.2
ESR Parameters for Metmyoglobin at 77 K

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$g_1$</th>
<th>$g_2$</th>
<th>$g_3$</th>
<th>$g_{av}$</th>
<th>$\Delta g$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 9.0</td>
<td>2.592</td>
<td>2.155</td>
<td>1.844</td>
<td>2.197</td>
<td>0.748</td>
</tr>
<tr>
<td>pH 9.0 (+E/G)</td>
<td>2.570</td>
<td>2.146</td>
<td>1.845</td>
<td>2.187</td>
<td>0.725</td>
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<tr>
<td>pH 10.5</td>
<td>2.583</td>
<td>2.169</td>
<td>1.873</td>
<td>2.208</td>
<td>0.710</td>
</tr>
<tr>
<td>pH 10.5 (+E/G)</td>
<td>2.601</td>
<td>2.110</td>
<td>1.862</td>
<td>2.191</td>
<td>0.739</td>
</tr>
</tbody>
</table>
TABLE 5.3

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Temp/K</th>
<th>$g_1$</th>
<th>$g_2$</th>
<th>$g_3$</th>
<th>$g_{av}$</th>
<th>$\Delta g$</th>
<th>h/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 8.0, 50%E/G.</td>
<td>77</td>
<td>2.41</td>
<td>2.11</td>
<td>1.95</td>
<td>2.16</td>
<td>0.46</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ca. 120</td>
<td>2.47</td>
<td>2.12</td>
<td>1.93</td>
<td>2.17</td>
<td>0.54</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ca. 155</td>
<td>2.57</td>
<td>2.17</td>
<td>1.85</td>
<td>2.20</td>
<td>0.72</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ca. 180</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.0</td>
</tr>
</tbody>
</table>

(Present Study)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Temp/K</th>
<th>$g_1$</th>
<th>$g_2$</th>
<th>$g_3$</th>
<th>$g_{av}$</th>
<th>$\Delta g$</th>
<th>h/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 9.0, 14%E/G.</td>
<td>77</td>
<td>2.43</td>
<td>2.12</td>
<td>1.93</td>
<td>2.16</td>
<td>0.50</td>
<td>-</td>
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<tr>
<td></td>
<td>ca. 155</td>
<td>2.49</td>
<td>2.14</td>
<td>1.91</td>
<td>2.18</td>
<td>0.58</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ca. 180</td>
<td>2.55</td>
<td>2.20</td>
<td>1.88</td>
<td>2.21</td>
<td>0.67</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>ca. 200</td>
<td>2.58</td>
<td>2.20</td>
<td>1.86</td>
<td>2.21</td>
<td>0.72</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>ca. 230</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(6.09/6.21)</td>
</tr>
</tbody>
</table>

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DISCUSSION

5.4.1 Reduction of Fe(IV) to Low Spin Fe(III)
Metmyoglobin and methaemoglobin undergo one-electron oxidation to give Fe(IV), the ferryl derivative. On exposure to $^{60}$Co $\gamma$-radiation, ferryl is reduced to a low spin Fe(III) compound at 77 K. On annealing the low spin Fe(III) changes to a high spin Fe(III). There are several intermediates in the transition between the first formed low spin Fe(III) and the terminal high spin Fe(III).

5.4.2 Structural Relaxation of the Low Spin Fe(III)
Inspection of the ESR parameters in Table 1 shows a gradual increase in $g_{av}$ from 2.16 for (a) to 2.21 for (d). A similar variation was obtained by Gasyna (1980). The steady departure from free spin indicates a spread of electron spin density from the ligand onto the iron centre. $\Delta g$ is also observed to increase generally with annealing. A correlation of both variations in $g_{av}$ and $\Delta g$ leads to the suggestion that electron delocalisation from the ligand is accompanied by an increase in bond lengths of some or all of the ligands. ESR parameters for Fe(III)(d) compare with the normal low spin Fe(III) in metmyoglobin at pH 10.5 which in fact is the hydroxy derivative, Fe(III)-OH. This is likely to have arisen from the radiation-reduced ferryl by the protonation of a structurally relaxed Fe(III)=O. The phenomenon of structural relaxation is in this case apparent from the multiple low spin forms obtained through electron delocalisation and bond lengthening.

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5.4.3 Structure of the Fe(IV) Complex

Resonance Raman spectroscopic studies by Sitter et al. (1985) indicate a shorter Fe-0 bond length in Fe(IV)=O than in Fe(III)-OH₂. Chance and coworkers (1986) have performed X-ray absorption studies (EXAFS) on a series of haemoprotein derivatives. Their results show that the oxo-myoglobin compound containing Fe(III)=O has an iron-oxygen bond shorter than in aquo-myoglobin containing Fe(III)-OH₂, the normal low spin Fe(III). It follows therefore that the ferryl derivative must be an oxo compound of the type Fe(IV) or [FeO]⁺.

5.4.4 Electron Addition to the Ferryl Complex

Electron-capture by the ferryl complex produces the low spin Fe(III) species 'a'. A graphical plot of the variation in g-values for a range of low spin Fe(III) species is shown in Figure 5.8. This plot is arbitrarily drawn to display g½ in a linear fashion, thus imposing some trend in the g₀ and g₃, both being indicators of crystal field characters. The species 'a' to 'd' show a gradual departure in their g-values away from free spin. In electronic terms, the higher the spin density towards the metal, the higher the spin orbit coupling imposed by the metal. This results in increasing Δg with 'a' 'd'. This behaviour resembles those found for α, γ and βδ species of FeO₂⁻ (Symons and Petersen, 1978).

It is likely in this case that the initial species 'a' is simply the electron adduct [FeO]⁺, retaining the strongly bound oxo ligand. On annealing, this forms a hydrogen bond to give the Fe(III)--OH⁻ low spin species which is later protonated to form the normal high spin Fe(III)--OH₂ species often observed.
Figure 5.8
Trends in the g-values for a range of low-spin Fe(III) complexes including FeO$_2^-$ and the ferryl-derived species
in acidic solutions of methaemoglobin or metmyoglobin.

5.4.5 The FeO\textsuperscript{2-} Species

On the irradiation products of oxyferrous myoglobin (or haemoglobin), the FeO\textsuperscript{2-} species obtained by Symons and Petersen (1978) have g-values quite different from those of Fe(III)'a' or any of the transition intermediates (see Table 1). Though FeO\textsuperscript{2-} also decays, on annealing, to high spin Fe(III), it is not identical with Fe(III)'a'. The g\textsubscript{nv} of FeO\textsuperscript{2-} and those of its relaxation species (α, β, γ, and δ), are so low as to suggest a lesser degree of electron delocalisation from the ligand to the metal. It is noted also that the first formed electron adduct species at 77 K goes through structural relaxation during annealing. The increase in g\textsubscript{nv} of successive species of Fe(III) from Fe(IV) (a → b → c → d) signifies electron delocalisation onto the metal, with increasing bond lengths. Within the scheme of related reactions FeO\textsuperscript{2-} may be considered as an unstable intermediate in the electron/proton addition reactions shown below:

\[
[\text{Fe(II)O}_2] = [\text{Fe(III)O}_2^-] \rightarrow [\text{Fe(II)O}_2^-] \quad \text{[1]}
\]

\[
[\text{Fe(II)O}_2^-] = [\text{Fe(III)O}_2^-] \rightarrow [\text{Fe(III)O}_2^\text{H}^-] \quad \text{[2]}
\]

\[
[\text{Fe(III)O}_2^\text{H}^-] \rightarrow \text{Fe(III)} + \text{H}_2\text{O}_2 \quad \text{[3]}
\]

If the species Fe(III)O\textsubscript{2}H\textsuperscript{-} is considered to be unstable, giving the ferryl complex with the release of OH\textsuperscript{-}

\[
[\text{Fe(III)O}_2^\text{H}^-]^{2+} \leftrightarrow [\text{Fe(IV)O}_2^\text{2-}]^{2+} + \text{OH}^\text{-} \quad \text{[4]}
\]

then there would have been a complete loss of all FeO\textsuperscript{2-} signals with a simultaneous secondary growth of OH\textsuperscript{-} during the
annealing procedure. This however was not the case.

The alternative consideration is for the loss of one electron from the porphyrin (whose orbital is well mixed-in with iron d_{xz} and d_{yz} orbitals);

\[ [\text{PFe(III)O}_2\text{H}^-]^2+ \rightarrow [\text{P'}^+\text{Fe(IV)O}^2^-]^3+ + \text{OH}^- \] \[ \text{[5]} \]

Whilst this may be possible in most haemoprotein reactions it probably constitutes a minor reaction compared to reaction [3] for haemoglobin and myoglobin. This suggestion derives from the fact that despite the shift in the soret region (412\rightarrow425nm) on adding hydrogen peroxide to methaemoglobin, the ESR signal at free spin does not reflect a quantitative conversion to the porphyryl-ferryl complex. For horseradish peroxidase it appears to constitute the major reaction. This difference may be in the structural distinction between the two classes of iron-proteins.

In the reactions with hydrogen peroxide,

\[ \text{Fe(III) + H}_2\text{O}_2 \rightarrow \text{Fe(III)O}_2\text{H}^- + \text{H}^+ \] \[ \text{[6]} \]

the initial product reacts with a second molecule of hydrogen peroxide;

\[ \text{Fe(III)O}_2\text{H}^- + \text{H}_2\text{O}_2 \rightarrow \text{Fe(IV)O}^2^- + \text{HO}_2^- + \text{H}_2\text{O} \] \[ \text{[7]} \]

These are thought to be the major reactions in haemoglobin and myoglobin. As for horseradish peroxidase, reaction [6] produces the porphyril radical cation as in reaction [5]. This then reacts with excess hydrogen peroxide as shown below;

\[ \text{P'}^+\text{Fe(IV)O}^2^- + \text{H}_2\text{O}_2 \rightarrow \text{PFe(IV)O}^2^- + \text{HO}_2^- + \text{H}^+ \] \[ \text{[8]} \]
Figure 5.9
ESR spectra at the free-spin region for the methaemoglobin-hydrogen peroxide complex at pH (a) 6.0, (b) 7.4, (c) 9.0
5.4.6 The Fenton Reaction

The Fenton reaction formally written as

$$\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{OH}^\cdot + \text{OH}^- \ldots \ldots \ldots \ldots [9]$$

may be presented as a two-part reaction involving the ferryl intermediate according to Walling (1975):

$$\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(IV)}\text{O}^2^- + \text{H}_2\text{O} \ldots \ldots \ldots \ldots [10]$$

$$\text{Fe(IV)}\text{O}^2^- + \text{H}_2\text{O} \rightarrow \text{Fe(III)} + \text{OH}^\cdot + \text{OH}^- \ldots \ldots \ldots [11]$$

These are possible reactions for free iron in solution, giving the hydroxyl radical as reported by Puppo and Halliwell (1987) for denatured haemoglobin. In the native protein however, the competitive formation of a porphyryl radical cation as shown in reaction [5], reduces the contribution of reaction [10], hence a diminution or virtual absence of the hydroxyl radical would be expected.

CONCLUSION

Haemoproteins react with hydrogen peroxide to produce the ferryl derivative which is an oxo complex, Fe(IV)=O. In acid, the ferryl complex is unstable, rapidly reacting with H\(^+\) to give high spin ferric with ESR parameters identical with those of acid methaemoglobin or metmyoglobin. Ferryl is reducible by \(\gamma\)-radiation to low spin Fe(III), at 77K. On annealing, the low spin product reacts with protons to form high spin Fe(III), like the one electron adduct, FeO\(^2-\) species.

The free radical produced in addition to the ferryl complex is probably a porphyryl radical. In the absence of the porphyrin as in denatured haemoglobin or free iron, a hydroxyl radical is the more likely free radical.
REFERENCES

6.1.1 INTRODUCTION

Chemical reactions which occur upon irradiation of aminoacids, peptides, and proteins are initiated primarily by the simultaneous loss and capture of electrons. The course of such reactions is determined by the types of radicals generated and their individual or interactive decay products. Centres which are most active for electron capture in metalloproteins are the metal chromophores. Others are the sulphur-bearing side groups and the aromatic and/or heterocyclic constituents. The selective protection of such electron-affinic groups by the input of external agents affords a preservation of gross structural features of the protein which would otherwise have been destroyed through irradiation. Structure is a strong determinant of physiological function. Its disruption inevitably results in the loss, or at the very least, impairment of function. On the other hand, the selective sensitisation of electron-affinic groups by the introduction of chemical modifiers may be beneficial in the mitigation of radiolytic damage to protein structures.

6.1.2 Genetic Implication of Electron and 'Hole' Transfer.

Interest in radiation biology in recent times has focussed on the nucleic acid components of the chromosome. This is because of its primary importance as a template for the transmission of genetic information to descendants. The major components of cell nuclei are deoxyribonucleic
acid (DNA) and the nucleoproteins, including the histones. Two forms of DNA are wrapped round an octet of histones called a nucleosome. Continuity between each nucleosome is achieved with a linear DNA and histone H1. Another nucleoprotein known to exist in large amounts in fish (salmon) sperm is protamine. Both the DNA and its associated proteins are prone to radiolytic attack by ionizing radiations.

In a series of publications (Huttermann et al. 1978; Cullis and Symons, 1986; Schulte-Frohlinde, 1986), the damage to DNA by direct radiation has been explained in terms of the production of the guanine radical cation, G•+, and thymine radical anion, T•−. The latter is protonated to give TH• above 130 K. Formation of both radicals represents the initiation of strand-break processes, which further implies deleterious effects on DNA replication. While a single strand-break (SSB) may be repairable, a double strand-break (DSB) is far less likely to be repaired. Indirect damage has to do with the interaction of OH• radicals (a primary product in the radiolysis of solvent water, see Section 5.3.1) with the DNA or nucleoproteins.

In broad terms, the phenomenon of radiolytic damage in biological systems depends on the mobility of both the electron and the 'hole' through the system. Furthermore the stability (or trapping) of emergent radicals may contribute to such radiolytic effects. This is dissimilar to electronic conduction in conductors and
semi-conductors, in which the 'hole' and the electron migrate without any interacting chemical species.

6.1.3 Radiation Effects in Proteins

Although radiation processes in proteins have been studied, attention has mainly centred on the role of the parent aminoacids, or the small oligopeptides (Sevilla et al. 1979; Garrison, 1987). The reactions in simple aminoacids are deamination and decarboxylation of the zwitterion:

\[
\begin{align*}
H_3^+\text{NCH(R)CO}_2^- + e^- &\rightarrow NH_3 + CH(R)CO_2^- \\
H_3^+\text{NCH(R)CO}_2^- &\rightarrow H_3^+\text{NCH(R) + CO}_2+e^- 
\end{align*}
\]

For the natural proteins experiments have been performed on the role of the variable side group R, especially those involving unsaturated rings and disulphide bridges (Swallow, 1977; Butler et al. 1982). In polyaminoacids, as in proteins, the end groups constitute only a very small minority. The major regular constitution is the polypeptide backbone comprising a long 'string' of amido groups as represented below:

![Polypeptide backbone diagram]

Figure 6.1
A segment of the polypeptide chain showing the string of amide linkages.
It is surprising that little attention has been given to the role of the amide chain in spite of its predominating presence.

Reactions [1] and [2] lead to the formation of a carbon-centred radical generally denoted $R^\cdot$. This is the terminal product of radiolysis in anaerobic systems. In aerobic systems the organic peroxy radical $RO_2^\cdot$ is the final product, arising from the reaction of $R^\cdot$ with dioxygen.

The simple aminoacids and the low molecular weight oligopeptides, especially their homopolymers, are much easier to analyse than the typical protein. In like terms, products from irradiation of homopolymers of aminoacids are simple to understand. They become more complex as the degree of polymerisation increases. Multiplicity of side chains will also complicate the analysis. The complex nature of a protein, typically comprising hundreds of aminoacid residues, and up to 19 different side groups, makes the identity of $R^\cdot$ quite ambiguous.

6.1.4 The Electron-loss Centre

Investigations by Symons and coworkers on nitrogen-carbon π-radicals (Lyons and Symons, 1972), amides and amide derivatives (Rao and Symons, 1982; Eastland et al. 1986), involved the generation of radical cations by γ-radiation of frozen aqueous solutions at 77 K. The radicals obtained were stable up to 160 K and therefore amenable to ESR
measurement with temperature variation. Their results clearly established the π-character of the primary radical cations. The SOMO (semi occupied molecular orbital) is localised, 75 ± 10%, on the nitrogen. The remainder is delocalised onto the oxygen of the carbonyl and the α-hydrogen.

In this study, it is proposed that the amido groups constitute the main centre of electron-loss, in line with results from amide radical cations. The primary product of γ-radiation is therefore a radical centred on the nitrogen of the ubiquitous peptide backbone. This undergoes conversion through hydrogen transfer, to a carbon-centred radical as the terminal product, (see section 6.3.4).
EXPERIMENTAL

6.2.1 Selection of Polyaminoacids and Proteins
The following samples were obtained from the Sigma Chemical Company:-- polyglycine, polyalanine, polyvaline, polytyrosine, protamine, mellitin, histone IIA, myoglobin and collagen. The samples were of the highest grade available and were used without further purification. Haemoglobin was isolated from whole blood by the method of Drabkin (1949).

6.2.2 Frozen Aqueous Solutions
Samples for irradiation were prepared by making a slurry of the dry compound (10mg) in 0.25ml of 1mM aqueous solution of tripotassioium hexacyanoferrate(III). The slurry was then frozen as pellets in liquid nitrogen. Irradiation was performed at 77 K in a $^{60}$Co $\gamma$-ray source at a dose rate of ca 0.6 Mrad/hr. At the later stages of this work, the slurry was dried by flushing with a slow stream of dry oxygen-free nitrogen gas (or air), having passed it through a column of dry silica gel. A flaky pale yellow powder was obtained. This was scraped and bottled for irradiation at 77 K. A regular dosage of 1.0 Mrad was used throughout this work.

Deoxygenated samples were prepared using a previously deoxygenated solution of the iron(III) salt. As much as practicable all subsequent procedure pre-irradiation was carried out in a nitrogen-filled glove box.
When deuterated samples were required the iron (III) solution was prepared \textit{ab initio} in D$_2$O.

ESR measurements with variable temperature facilities were made as outlined in Section 4.2.2 and 4.2.3.

6.2.3 Comments on Powder Samples

It was found desirable to eliminate or at least reduce the OH' (or OD') signal which was found to dominate the spectrum at 77 K. The benefit of this was to enhance the radical detection and also to elicit the low-field ESR features of radicals produced at 77 K. A high degree of dehydration and yet the presence of the electron scavenger suitably attached to the protein or polypeptide was attempted. The use of pastes did decrease the amount of solvent in frozen samples. The drying to flakes virtually eliminated solvent radicals just as expected.

It is pertinent to note however that drying of pastes consumed large volumes of nitrogen over very prolonged time duration. The eventual quantity of flake was also very small. At least four rounds of flake preparation were required for one experiment. Further to this, the drier the sample the greater the electrostatic-induced scattering of the powder as was observed during packing for irradiation. 'Sample-floating' in liquid nitrogen was also difficult to overcome. The latter phenomenon was thought to be responsible for the very noisy spectra obtained through this attempt. For these reasons the procedure of special dessication to flakes was not much favoured.
RESULTS AND DISCUSSION

6.3.1 Polyaminoacids

Fig. 6.2(a) shows the ESR spectrum obtained for polyglycine after γ- irradiation at 77 K. The spectrum shows a predominance of OH' radical at 77 K. Annealing results in the decay of OH' radical with the preservation of a doublet spectrum. The annealing sequence spectrum Fig. 6.2(b) shows that the doublet signal was produced at 77 K. The 1:1 doublet spectrum is shown more clearly in Fig. 6.2(c). From the structure of polyglycine the only source of a doublet spectrum is the α-carbon atom with the hydrogen side group. The loss of one of the hydrogens on the α-carbon either by 1-2 hydrogen transfer or intermolecular hydrogen transfer, is implied by this result.

The formation of a similar carbon-centred radical had been reported by Sevilla and coworkers (1979). Their discussion concentrated on primary deamination and decarboxylation, followed by hydrogen abstraction from the α-hydrogen on the polypeptide chain. A doublet splitting of 18-19G, due to one hydrogen on the carbon-centred radical was obtained. The doublet obtained in the present study is ascribed to an identical radical though the mechanism of its formation is here proposed to be different, as presented in sections 6.3.2 and 6.3.3.

In polyalanine the side group is a methyl group. The
ACTION OF IONIZING RADIATION ON WATER

\[ \text{H}_2\text{O} \xrightarrow{\gamma} \text{e}_{\text{aq}}, \text{H}^+, \text{OH}^-, \text{H}_3\text{O}^+, \text{H}_2, \text{H}_2\text{O}_2 \]

**Radical Products:**

\[ \text{H}_2\text{O} \xrightarrow{\gamma} \text{H}_2\text{O}^+ + \text{e}^- \]
\[ \text{e}^- + \text{nH}_2\text{O} \xrightarrow{} \text{e}_{\text{aq}} \]
\[ \text{H}_2\text{O}^+ + \text{H}_2\text{O} \xrightarrow{} \text{OH}^- + \text{H}_3\text{O}^+ \]
\[ \text{H}_2\text{O} \xrightarrow{} \text{H}_2\text{O}^* \xrightarrow{} \text{H}^+ + \text{OH}^- \]
\[ \text{e}_{\text{aq}} + \text{H}_3\text{O}^+ \xrightarrow{} \text{H}^+ + \text{H}_2\text{O} \]

**Molecular Products:**

\[ \text{H}^+ + \text{H}^+ \xrightarrow{} \text{H}_2 \]
\[ \text{e}_{\text{aq}} + \text{e}_{\text{aq}} \xrightarrow{2\text{H}_2\text{O}} \text{H}_2 + 2\text{OH}^- \]
\[ \text{e}_{\text{aq}} + \text{H}^+ \xrightarrow{\text{H}_2\text{O}} \text{H}_2 + \text{OH}^- \]
\[ \text{OH}^- + \text{OH}^- \xrightarrow{} \text{H}_2\text{O}_2 \]

**Yields:**

Species: \( \text{e}_{\text{aq}}, \text{H}^+, \text{OH}^-, \text{H}_3\text{O}^+, \text{H}_2, \text{H}_2\text{O}_2 \)

G-values: 2.7 0.55 2.7 2.7 0.45 0.7

\((G = \text{No. of molecules produced per 100eV})\)
**Figure 6.2(a)**
First derivative X-band spectrum for $\gamma$-irradiated frozen aqueous solution of polyglycine showing the predominant OH$^-$ radical features at 77 K.
Figure 6.2(b)
First derivative X-band spectra for γ-irradiated frozen aqueous solution of polyglycine showing progressive loss of OH\(^\cdot\) features during annealing from 77 K to ca. 130 K.
Figure 6.2(c)

First derivative X-band spectrum for γ-irradiated frozen aqueous solution of polyglycine annealed from 77 K to ca. 130 K.
spectrum obtained on irradiation at 77 K is shown in Fig. 5.3(a). This also shows a predominating OH' radical, as well as wing features. After annealing to remove the OH' radical (at ca. 130 K), two species are discernible in Fig. 5.3(c). One is a four-line spectrum with the intensity ratio of 1:3:3:1. The other, in a much smaller amount, is ascribed to a nitrogen-centred radical cation being part of the electron-loss centre (as discussed later in this section). The annealing sequence spectra in Fig. 5.3(b) show that the two surviving radicals were both produced at 77 K. They might have been produced simultaneously or consecutively. The major quartet signal must have arisen from the -CH₃ side group and only if the hydrogen on the polypeptide chiral carbon centre was lost. This result is in line with deductions by Snipes and Schmidt (1966), on the formation of -N(H)-C'(CH₃)-C(O)- after hydrogen abstraction from the main α-carbon.

Polytyrosine has a 4 methyl phenoxy side group. On irradiation, it undergoes two distinct reactions. One is electron-loss from the aromatic ring, to produce a tyrosyl radical cation which loses a proton as follows:

\[
\begin{align*}
\text{I} & \quad \text{II} & \quad \text{III} \\
\text{CH}_2 & \quad \text{CH}_2 & \quad \text{CH}_2 \\
\text{OH} & \quad \text{OH} & \quad \text{O}^- \\
-e^- & \quad \text{loss of proton} & \quad + H^+ \\
\end{align*}
\]

The terminal product III has an intense ESR signal centred
Figure 6.3(a)

First derivative X-band spectrum for γ-irradiated frozen aqueous solution of polyalanine showing the predominant OH' radical feature at 77 K.
Figure 6.3(b)

First derivative X-band spectra for γ-irradiated frozen aqueous solution of polyalanine showing progressive loss of OH' features during annealing from 77 K to ca. 130 K.
Figure 6.3(c)

First derivative X-band spectrum for γ-irradiated frozen aqueous solution of polyalanine annealed from 77 K to ca. 130 K.
at free-spin with a line width of 18G as seen in Fig.6.4. This is in line with the results of King and others (1964). The other reaction is electron-capture by the phenoxy ring to give a radical anion which probably undergoes 1-3 hydrogen transfer thus:

![Chemical structure diagram](image)

The ESR spectrum in Fig.6.4 is due to the aryloxyl radical III with contributions from the two hydrogens at the 2-position (ca. 45G each), the 3- and 5- hydrogens (ca. 10G each), and 6- position hydrogen (ca. 6G). This analysis is in line with that of Liming and Gordy (1968) on the comparison of radical features from polytyrosine and polyphenylalanine.

### 6.3.2 The Electron-loss Centre

The spectrum shown in Fig.6.6 is the electron-loss centre in methaemoglobin; electron-capture having taken place at the Fe(III) centre. The planar peptide (amide) unit
Figure 6.4
First derivative X-band spectrum for γ-irradiated frozen aqueous solution of polytyrosine after annealing to ca. 130 K.
is the ubiquitous backbone of the protein, and is expected to lose an electron readily as is the case for simple amides. The radical cation (-CONH\(^+\)-CHR-) can readily lose the N-H protons via hydrogen-bonding. The electron-loss centre thus becomes fixed, and gives a detectable ESR spectrum. The following ESR features are expected by comparison with a wide range of nitrogen-centred radicals:

(a) Relatively weak \( M(\text{^14N}) = \pm 1 \) 'parallel' features together with an intense nearly isotropic \( M(\text{^14N}) = 0 \).

(b) Each of these features should be split into nearly isotropic doublets by coupling to the unique \( \beta \)-protons of the -CHR- unit attached to nitrogen.

The difficulty with this prediction is that the magnitude of the coupling to \( \beta \)-protons is a function of the angle \( \Theta \), defined below;

\[ \theta \]

\[ 2p \text{orbital} \]

\[ \text{C-H bond} \]

\[ \text{N} \]

\[ \text{R} \]

\[ \text{X} \]

\[ \text{Y} \]

\[ \text{Z} \]

\[ \text{H} \]

\[ \text{CO} \]

**Figure 6.5**

View along the N-C bond with the amide unit in the X-Y plane, showing the angle \( \Theta \) between the 2\( p_z \) orbital on N and the C-H bond.
The coupling of the \( \beta \)-protons to the nitrogen is expressed in terms of the dihedral angle \( \Theta \) by the equation
\[
A(\beta H) = A_{\text{max}} \cos^2 \Theta \quad \text{[3]}
\]
where \( A_{\text{max}} \) is ca. 50G, and \( 0 \leq \Theta \leq 90^\circ \).

Therefore \( A(\beta H) \) could take any values from ca. 50G to zero, depending on \( \Theta \). Absence of any proton splitting suggests that \( \Theta = 90^\circ \), giving \( A(\beta H) = 0 \). The maximum proton splitting occurs when the \( \beta \)-hydrogen aligns with the nitrogen p-orbital to give \( \Theta = 0^\circ \), and \( A(\beta H) = 50G \).

A plot of angle of twist of the polypeptide coil, as an index of secondary structure, versus the dihedral angle \( \Theta \), for many proteins shows that values of \( \Theta \) tend to cluster for secondary structures which are consistent and predominant (Ramachandran and Sasisekharan, 1968). For the \( \alpha \)-helix the average value of \( \Theta \) is 30\(^\circ\). Substituting this value of \( \Theta \) into equation [3] gives a coupling of 38G, being the expected coupling for an \( \alpha \)-helical secondary structure.

Calculations from the data of Pauling and Corey (1951), and Kendrew (1962), give a value of 73\% as the \( \alpha \)-helical content of haemoglobin. ESR results for methaemoglobin, Fig. 6.6, oxyhaemoglobin and deoxyhaemoglobin/[Fe(CN)\(_6\)]\(^{3-}\), Fig. 6.7, are in agreement with these calculations. In these proteins electrons are captured by the iron units, so that the \( g = 2 \) region of the spectrum is dominated by the electron-loss centre. The results show that the major species has a large \(^{14}\text{N} \) parallel splitting (ca. 43G) but a
Figure 6.6
First derivative X-band spectrum for methaemoglobin after γ-irradiation at 77 K, and annealing to ca. 130 K, showing features assigned to the electron-loss centre.
Figure 6.7
First derivative X-band spectrum for deoxyhaemoglobin/[Fe(CN)$_6$]$^{3-}$ complex after \(\gamma\)-irradiation and annealing to \(\approx 130\) K, showing features assigned to the electron-loss centre.
small $\beta$-proton coupling (0±5G). This accords well with an 
$\alpha$-helical structure which in fact should give $A(\beta H) = 38G$.
Weak outer features and a central ($M_i = 0$) doublet with 
$A(\beta H)$ ca. 38G can be seen. As indicated, this splitting 
quite accommodates the outer parallel, lines given that as 
expected, $A'^{14}N$ is ca. 43G. This is the upper limit 
presented in the Ramachandran plot.

A simulation of ESR spectrum with these parameters is 
shown in Fig.6.8. The good match between experimental and 
simulated spectra confirms the above prediction.

Similar results were obtained for other proteins in which 
there are significant amounts of $\alpha$-helical structure. 
Fig.6.9 shows the spectrum of the electron-loss centre in 
protamine, the nucleoprotein found in fish (salmon) sperm. 
The predominant conformation here is the $\alpha$-helix, 
(Dayhoff, 1972). This spectrum shows the expected outer 
parallel splittings, $A(\beta H)$ of ca. 42G, which conforms with 
the prediction as well as the synthesised spectrum in 
Fig.6.8. Collagen, the triple helix protein (Bornstein and 
Traub, 1979), also gives in Fig.6.10, the expected result 
in line with the same prediction.

Other proteins examined include histone IIA (from calf 
thymus), melittin (from bee venom), lysozyme (from egg 
white), and serum albumin (from bovine). In these cases 
the electron-loss centres were not as well defined 
spectrally, as the the synthesised spectrum or the earlier 
selected samples. The reason for this is ascribed to the
Figure 6.8
Computer synthesised ESR spectrum for a combination of two amide radicals, both having $A(^{14}\text{N}) = 42\text{G}$, one having $A(^{1}\text{H}) = 0$, and the other having $A(^{1}\text{H}) = 38\text{G}$. 
Figure 6.9
First derivative X-band spectrum for protamine/[Fe(CN)$_6$]$^{3-}$ complex after $\gamma$-irradiation and annealing to $\approx 130$ K, showing features assigned to the electron-loss centre.
Figure 6.10
First derivative X-band spectrum for collagen/[Fe(CN)$_6$]$_{5-}$ complex after γ-irradiation and annealing to ca. 130 K, showing features assigned to the electron-loss centre.
presence of significant amounts of sulphur-bearing side
groups in lysozyme and bovine serum albumin, and the
presence of aromatic side groups in histone and melittin.
These side groups give ESR features which overlap proton
splittings of the peptide hydrogen. In each case,
electron-capture was by an external scavenger in the form
of hexacyano iron(III) or internal electron sinks like
endogeneous iron (III) or -S-S- groups. Other radical
cations with intermediate Θ-values arising from the
disordered non-helical regions of the proteins contribute
to the background in these spectra.

6.3.3 The β-Sheet Structure

Next to the α-helix, the secondary structure of widespread
occurrence in proteins is the β-sheet. In accordance with
the IUPAC-IUB Commission (1970), the dihedral angle in the
β-sheet is larger than that in the α-helix. For the
β-sheet, Θ is 60°. Using the prediction above, a nitrogen
parallel splitting A(βΗ) of 12.5G, as the outer feature
in the ESR spectrum is expected. For this structure,
α-chymotrypsin was found suitable for its high β-sheet
content (Birktoft and Blow, 1972). The spectrum obtained
for the electron-loss centre is shown in Fig.6.11. An
outer parallel feature of 12G is obtained here as
predicted. On comparison with the simulated spectrum in
Fig.6.12, a fairly good agreement is obtained. The
simulated spectrum however contains A(βΗ) of 14.5G,
corresponding to Θ = 57.4°, a difference of 2.6°. This
difference is well accommodated within the Ramachandran
plot.

-119-
Figure 6.11
First derivative X-band spectrum for $\alpha$-chymotrypsin/[Fe(CN)$_6$]$^{3-}$ complex after $\gamma$-irradiation and annealing to ca.130 K, showing features assigned to the electron-loss centre.
Figure 6.12

Computer synthesised ESR spectrum for a combination of two amide radicals, both having $A({}^1\text{N}) = 42\text{G}$, one having $A({}^1\text{H}) = 0$, and the other having $A({}^1\text{H}) = 14.5\text{G}$.
6.3.4 Hydrogen Transfer Schemes.

The nitrogen-centred radical formed at the amide group may undergo a hydrogen transfer reaction to give a carbon-centred radical as the product. There are two possible pathways for the hydrogen transfer. One is the intermolecular hydrogen transfer in which a hydrogen is transferred from a neighbouring side group to the nitrogen centre as shown below:

The other scheme involves the transfer of the hydrogen on the alpha carbon to the nitrogen centre; a form of 1-2 hydrogen shift:

The results obtained for synthetic polyaminoacids do not show the nitrogen-centred radical in any regular
conformation within the polypeptide twist. This is not surprising in view of the discrepancy in the twisting of the polypeptide chain compared with the regularity in natural proteins. The nitrogen-centred radical formed in the polyaminoacids may therefore have an ESR spectrum which reflects the sum of all possible \( \beta \)-proton couplings for \( 0 \leq \theta \leq 90^\circ \). A broadening of the ESR peaks is therefore the end result. One consistent aspect of the structures is the occurrence of the same side group. It thus stands to reason that most of the assignable features should originate from the regular side groups in homopolymers. This explains the predominant contribution from the side groups as obtained.
CONCLUSION

For the proteins studied, electrons ejected by ionising radiation have significant mobility and are effectively captured at electron-affinic sites. These may be internal agents like the endogenous metal, aromatic, or \(-\text{S-S-}\) side groups. The 'holes' originate primarily from the peptide amido groups and are rapidly trapped, by loss of the N-H proton, as nitrogen-centred radical cations. The trapped 'hole' has been here characterised by ESR spectroscopy.

The variable \(\beta\)-proton coupling to the amido nitrogen p-orbital provides basis for the characterisation of protein secondary structures. This is based on the regularity of dihedral angles throughout major segments of a protein coil. This application of the ESR technique is a departure from conventional methods like circular dichroism, X-ray crystallography, proton NMR, and electron microscopy popularly used for stuctural determinations.

With respect to radiation damage in the cell nuclei, electrons ejected from the protein migrate to the associated DNA. This results in a significant increase in the yield of the electron-capture product, \(T^-\) (Cullis et al. 1987). The 'hole-centres' in the protein are trapped and do not lead to any detectable increase in the yield of \(G'^+\).
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IUPAC-IUB Commission on Biomedical Nomenclature 1969 (1970), Biochem. 9, 3471


Chapter 7

Electron-Transfer in Xanthine Oxidase
7.1.1 INTRODUCTION

Xanthine Oxidase is a member of the group of enzymes known as molybdenum iron-sulphur flavin hydroxylases. Others are aldehyde oxidase, and xanthine dehydrogenase. Generally, they catalyse the hydroxylation of purines, pyrimidines, and pteridines. Xanthine oxidase in particular catalyses the hydroxylation of xanthine to uric acid in a two-electron oxidation reaction;

\[
\begin{align*}
\text{Xanthine} & \quad \text{Uric acid} \\
\text{(2,6 dihydroxypurine)} & \quad \text{(2,6,8 trihydroxypurine)}
\end{align*}
\]

Xanthine oxidase, in its catalytic action facilitates the above oxidation through the acceptance of two electrons followed by a series of intramolecular electron transfer reactions. The enzyme finally completes a turn-over cycle (complete reduction and reoxidation), when the electrons are discharged onto the oxidising substrate, in this case oxygen. The series of intramolecular electron transfer reactions constitute the enzymic mechanism to which this work is addressed.

7.1.2 Structure

Bovine milk xanthine oxidase is a dimer of two
structurally identical monomers, each of relative molar mass 140,000. Each monomer comprises one molybdenum centre, one flavin, four irons and four acid-labile sulphurs (Massey et al. 1969). The iron and sulphur constitute two pairs of iron- sulphur clusters \((\text{Fe}_2\text{S}_2)\), labelled \(\text{Fe/ }\text{S(I)}\) and \(\text{Fe/ }\text{S(II)}\), which may be structurally identical but functionally differentiated (Lowe et al. 1972).

In its resting form, the active enzyme is fully oxidised, i.e. molybdenum is in the +6 oxidation state, the irons are in the +3 oxidation state, and the flavin exists as flavin adenine dinucleotide (FAD). These are all attached to the polypeptide backbone of which the protein is constituted. A gross structural representation is shown below:

```
| Mo(VI) |
| S-Fe-Fe-S | |
| S-Fe-Fe-S II |
| FAD |
```

Bray (1975) estimated from magnetic coupling interactions, the separation between the molybdenum and iron-sulphur centres to be of the order of 30 Å. In subsequent studies however (Love and Bray, 1978) the distance has been put at 25 Å for the bovine milk derived enzyme. Earlier studies on aldehyde oxidase by Rajagopalan and coworkers (1968) showed the presence of a paramagnetic centre at 10 to 20 Å from the flavin. The identity of this centre is yet to be ascertained. Since it could be either of the two irons in
the pair of iron-sulphur centres or the molybdenum an acceptable electron transfer sequence through the molecule may reveal the true spatial arrangement of the four centres. An unambiguous assignment however rests with X-ray crystallography.

7.1.3 The Catalytic Mechanism

During catalysis the reducing substrate, xanthine, loses two electrons to the enzyme in a one-step oxidation/reduction process during which the molybdenum is first reduced from +6 to the +4 oxidation state. The two electrons are later distributed to the iron-sulphur centres and the flavin by intramolecular electron transfer. A fully reduced enzyme may be represented thus:

\[
\begin{align*}
\text{Mo(IV)} & \\
\text{S-Fe(II)-Fe(III)-S(I)} & \\
\text{S-Fe(II)-Fe(III)-S(II)} & \\
\text{FADH}_2 & 
\end{align*}
\]

Between the fully oxidised and fully reduced forms, there is a series of equilibria involving species in which the centres are in intermediate degrees of oxidation. It is in this scheme that the ESR observable Mo(V) arises. For the complete reduction of an enzyme monomer six electrons are required; two add to the molybdenum, one adds to each of the irons, and two add to the flavin. Hille and Massey (1982) obtained an eight-electron reduced specie using dithionite. The difference of two electrons was reported to have been added to disulphide groups on the protein.
Rao *et al.* (1983) have since reported that disulphide groups are efficient electron scavengers during radiolysis. It is surprising therefore that the direct participation of disulphide groups in the catalytic mechanism has since not been observed or does in fact not exist. It is also surprising that in the reduction of iron centres only one of the constituent irons in each cluster is reducible, the other remaining in its native +3 state. This property is similar to single electron capture by the dicopper units in the haemocyanins (Symons and Petersen, 1978).

On the sequence of intramolecular electron transfer, the first suggestion was made by Bray and coworkers (1963) using a combination of rapid-freeze and ESR techniques. A time-dependent sequence of appearance of ESR signals was associated with progressive electron mobility through the centres in the linear order;

\[
\text{Xanthine} \rightarrow \text{Molybdenum} \rightarrow \text{FAD} \rightarrow \text{Fe/S} \rightarrow \text{O}_2
\]

Later studies by Komai and others (1979), and Edmondson and coworkers (1973), pointed at a non-linear model of the type

\[
\text{Xanthine} \rightarrow \text{Mo} \rightarrow \text{Fe/S} \rightarrow \text{O}_2
\]

The presence of the oxidising substrate, oxygen, is a
prerequisite for the enzyme to turn-over.

In the reoxidation of a six-electron reduced enzyme there is a succession of two 2-electron oxidation steps. The last two electrons are discharged in two single-electron steps. While the first two steps produce two peroxide ions, the last two steps result in the formation of two molecules of the superoxide anion (Olson et al. 1974). The proposed scheme is as shown below:

\[ \begin{align*}
X_0 & \xrightarrow{H_2O_2} X_2 \\
X_2 & \xrightarrow{H_2O_2} X_0
\end{align*} \]

The subscripts 0 to 6 indicate the degree of reduction, i.e. number of reducing electrons present in the molecule. All oxidation steps in the above scheme are fast except for the last one which is reportedly slow.

Edmondson and coworkers (1973) showed by pH jump studies that the rate of intramolecular electron transfer is faster than the rate of enzyme turn-over. This implies that the series of transient partially reduced intermediates are all present in the course of a catalytic process. The number of constituent species presumed present during the reoxidation of a six-electron reduced enzyme has been worked out by Olson and coworkers (1974)
to be thirty-six. In the real sense however only a two-electron reduced enzyme is involved in the hydroxylation of xanthine.

7.1.4 Participation of Molybdenum during Catalysis

The oxidation state of molybdenum in the resting enzyme is +6. During catalysis the equilibrium between +6 and +4 affords the presence of a +5 state. Mo(V); $4d^1, s=1/2$ is paramagnetic, a property which makes it amenable to detection by ESR. Bray (1961) detected a reversibility between the +5 and +6 states. Massey et al. (1970) later provided evidence for the presence of Mo(IV) in equilibrium during turn-over. The question therefore arises, that, when the intensity of the ESR signal of Mo(V) diminishes, is it due to its further reduction to Mo(IV) or reoxidation to Mo(VI)? Neither Mo(IV) nor Mo(VI) is detectable by ESR. Mention has also been made of Mo(III), $s=3/2$ arising from Mo(IV) (Bray, 1974). The absence of a four-line ESR spectrum however eliminates this possibility.

7.1.5 Types of Mo(V) ESR Signals

Various types of Mo(V) signals have been obtained with different chemical reducing substrates. The stability of some of these is fairly high, thus resulting in the overlap of decaying signals with emergent ones in a continuous reaction sphere. Four main types of molybdenum ESR signals have been identified (Bray and Swann, 1972). These have been labelled 'Very Rapid', 'Rapid', 'Slow',
and 'Inhibited'. They are all characterised by different g-values in their respective ESR spectra. The Very Rapid centre is ascribed to a first-formed transient intermediate appearing in the initial 10 ms. of the enzymatic reaction (Gutteridge and Bray, 1980). It decays to the Rapid species within 200 ms. Gutteridge and Bray proposed that the reducing substrate first complexes with the enzyme at the molybdenum terminal oxo group to form a Mo-O-C structure. The carbon atom involved had earlier been determined to be the C-8 position on the xanthine, by $^{13}$C-splitting of Mo(V) Very Rapid signal (Gutteridge et al. 1978a). Following the formation of the Very Rapid centre, the hydrogen from C-8 is rapidly transferred to a terminal sulphur ligand on the molybdenum. The Mo-SH structure gives rise to the Rapid signal (Gutteridge et al. 1978b). This accords with the presence of terminal sulphur in the active enzyme, and its absence in the desulpho inactive variant, as earlier reported by Bordas et al. (1979) and Tullius et al. (1979).
EXPERIMENTAL

7.2.1 Sample Preparation

Xanthine oxidase isolated from fresh bovine milk by the method of Massey et al. (1969) was kindly provided by Dr. Russ Hille (Ohio State University). The enzyme was supplied in 100mM pyrophosphate, pH 8.5. All experiments were performed with the stock solution of the enzyme without further purification.

The enzyme was deuterated by three cycles of concentration by vacuum dialysis and dilution with D₂O at 277 K.

γ-irradiation of frozen aqueous solutions of xanthine oxidase was performed at 77 K in a ⁶⁰Co γ-ray source. ESR measurements were carried out as explained in Sections 4.2.2 and 4.2.3. Measurements below 77 K were performed using a liquid helium cryostat (Oxford Instruments ESR 900), with the temperature indicator and controller DTC2.
RESULTS

7.3.1 Very Rapid Mo(V) and Fe/S(I)

Exposure of frozen aqueous solution of xanthine oxidase to $^{60}$Co $\gamma$-radiation at 77 K gave two main features identified as the Very Rapid Mo(V) and the reduced iron-sulphur centre(I), Fe/S(I), shown in Fig.7.1. g-Values obtained from this spectrum are the respective $g_2$ and $g_3$ for both centres. The $g_1$ signals are concealed beneath the predominant OH* signal produced in the solvent water, (see Section 6.3.1). Other features concealed are the electron-loss centre in the protein, radicals from buffer constituents, and the flavin radical, FADH*. g-Values calculated from Fig.7.1 are, for the Very Rapid Mo(V): $g_2 = 1.953$, and $g_3 = 1.950$ and for the reduced Fe/S(I): $g_2 = 1.935$, $g_3 = 1.896$.

On annealing above 77 K, changes were observed in the spectra. The predominant OH* radical decayed above 130 K to reveal two features at $g = 2.042$ and 2.023, Fig.7.2. These are the $g_1$ signals for Fe/S(I) and Very Rapid Mo(V) respectively. The intensities of these two features surprisingly do not correspond to those of their low field components. The huge isotropic signal centred at about free spin is thought to contain the FADH* radical signal. At ca. 180 K the Very Rapid Mo(V) has almost disappeared while the Fe/S(I) is still prominent, Fig.7.3. Further annealing to ca. 263 K shows the appearance of features ascribed to the Rapid Mo(V), Fig.7.4. The reduced Fe/S(I)
Table 7.1

ESR Parameters for various Mo(V) Centres in reduced Xanthine Oxidase

<table>
<thead>
<tr>
<th>Centre</th>
<th>Reductant</th>
<th>$g_1$</th>
<th>$g_2$</th>
<th>$g_3$</th>
<th>$g_{av}$</th>
<th>$A_1$</th>
<th>$A_2$</th>
<th>$A_3$</th>
<th>$^1\text{H}\text{ hfc}/G$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo(V)</td>
<td>Xanthine</td>
<td>2.025</td>
<td>1.956</td>
<td>1.951</td>
<td>1.977</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(a)</td>
</tr>
<tr>
<td></td>
<td>$e^-(\gamma\text{-rays})$</td>
<td>2.025</td>
<td>1.956</td>
<td>1.951</td>
<td>1.977</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(b)</td>
</tr>
<tr>
<td></td>
<td>(in $H_2O$) $e^-(\gamma\text{-rays})$</td>
<td>2.025</td>
<td>1.953</td>
<td>1.950</td>
<td>1.976</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(c)</td>
</tr>
<tr>
<td></td>
<td>(in $D_2O$) $e^-(\gamma\text{-rays})$</td>
<td>2.025</td>
<td>1.953</td>
<td>1.950</td>
<td>1.976</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(c)</td>
</tr>
<tr>
<td>Mo(V)</td>
<td>Xanthine(1)</td>
<td>1.989</td>
<td>1.969</td>
<td>1.964</td>
<td>1.974</td>
<td>12.4</td>
<td>12.0</td>
<td>12.0</td>
<td>(a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xanthine(2)</td>
<td>1.994</td>
<td>1.968</td>
<td>1.961</td>
<td>1.974</td>
<td>12.1</td>
<td>12.1</td>
<td>12.1</td>
<td>(a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Salicyl.(A)</td>
<td>1.991</td>
<td>1.968</td>
<td>1.963</td>
<td>1.974</td>
<td>14.3</td>
<td>14.0</td>
<td>14.0</td>
<td>(a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Salicyl.(B)</td>
<td>1.991</td>
<td>1.966</td>
<td>1.963</td>
<td>1.973</td>
<td>14.3</td>
<td>14.0</td>
<td>14.0</td>
<td>(a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$e^-(\gamma\text{-rays})$</td>
<td>1.990</td>
<td>1.969</td>
<td>1.965</td>
<td>1.975</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
<td>(b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(in $H_2O$) $e^-(\gamma\text{-rays})$</td>
<td>1.990</td>
<td>1.967</td>
<td>1.962</td>
<td>1.973</td>
<td>15.2</td>
<td>16.0</td>
<td>15.6</td>
<td>(c)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(in $D_2O$) $e^-(\gamma\text{-rays})$</td>
<td>1.994</td>
<td>1.966</td>
<td>1.961</td>
<td>1.974</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(c)</td>
<td></td>
</tr>
</tbody>
</table>

Ref.

(a) Bray, R.C. (1974)
(b) Symons, M.C.R. and Petersen, R.L. (1978)
(c) This study

$^1\text{H}\text{ hfc}$ is proton hyperfine coupling.

Salicyl. is salicylaldehyde

e$^-$ is dry electrons from a $^{60}\text{Co}\gamma$-ray source.
### Table 7.2

ESR Parameters of reduced Fe/S(I) and Fe/S(II) Centres in Xanthine Oxidase

<table>
<thead>
<tr>
<th>Centre</th>
<th>Reductant</th>
<th>( g_1 )</th>
<th>( g_2 )</th>
<th>( g_3 )</th>
<th>( \bar{g} )</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>e/S(I)</td>
<td>e(^{-}) ((\gamma)-rays)</td>
<td>2.045</td>
<td>1.925</td>
<td>1.890</td>
<td>1.953</td>
<td>(b)</td>
</tr>
<tr>
<td></td>
<td>( S_2O_4^{2-} )</td>
<td>2.022</td>
<td>1.935</td>
<td>1.899</td>
<td>1.952</td>
<td>(d)</td>
</tr>
<tr>
<td></td>
<td>e(^{-}) ((\gamma)-rays)</td>
<td>2.044</td>
<td>1.935</td>
<td>1.896</td>
<td>1.958</td>
<td>(c)</td>
</tr>
<tr>
<td>e/S(II)</td>
<td>e(^{-}) ((\gamma)-rays)</td>
<td>2.120</td>
<td>2.007</td>
<td>1.910</td>
<td>2.012</td>
<td>(b)</td>
</tr>
<tr>
<td></td>
<td>( S_2O_4^{2-} )</td>
<td>2.120</td>
<td>2.007</td>
<td>1.910</td>
<td>2.012</td>
<td>(d)</td>
</tr>
<tr>
<td></td>
<td>e(^{-}) ((\gamma)-rays)</td>
<td>2.120</td>
<td>2.007</td>
<td>1.904</td>
<td>2.010</td>
<td>(c)</td>
</tr>
</tbody>
</table>

**Ref.**

a) Bray, R.C. (1974)
c) This study
d) Lowe, D.J. *et al.* (1972)
Figure 7.1
First derivative X-band spectrum of xanthine oxidase after g-irradiation at 77 K showing features assigned to the Very Rapid Mo(V) and reduced Fe/S(I)
Figure 7.2
Spectrum of irradiated xanthine oxidase after annealing to ca.138 K, showing $g_1$ features for reduced Fe/S(I) and Very Rapid Mo(V)
Figure 7.3
Spectrum of irradiated xanthine oxidase after annealing to ca. 180 K, showing persistence of reduced Fe/S(I) after the loss of VR Mo(V)
has now disappeared. The g-values obtained for the Rapid are: 1.990, 1.967, and 1.962. The respective proton splittings are 15.2, 16.0, and 15.6G. Hyperfine splittings resulting from $^{95}$Mo are observed distinctly in the high field region in Fig. 7.4.

In order to eliminate proton splittings and also enhance the major signals due to Mo(V), the experiment was repeated with a deuterated sample of xanthine oxidase. Apart from the formation of OD' (instead of OH') radicals there was no difference in the spectra between 77 K and 180 K when OD' radicals had decayed and the Rapid Mo(V) formed. Fig. 7.5 shows the spectrum of the deuterated analogue after annealing to ca. 263 K and refreezing to 77 K. The g-values obtained for the Rapid Mo(V) are 1.994, 1.996, and 1.961. These are essentially the same as for the H$_2$O sample. It is important to note here that in the process of deuteration, the buffer concentration was reduced to 1% of its original value. In consequence the ionic strength was lower. It would not be expected however that the pH of the resulting solution would differ from the pH of its original stock. Hyperfine splittings due to $^{95}$Mo was found to be 24 Gauss on the three axes.

The presence of $^{95}$Mo hyperfine splittings for the Very Rapid signal was not easily detectable at 77 K because of the dominant signal of Fe/S(I) in the high field region, a position where the splittings should normally occur. The low field region is of course dominated by OH' signal.
Figure 7.4
Spectrum of irradiated xanthine oxidase after annealing to ca. 263 K, showing features assigned to the Rapid Mo(V) species with $^{95}$Mo hyperfine splittings
Figure 7.5
Spectrum of deuterated xanthine oxidase after irradiation and annealing to ca. 263 K, showing features for the Rapid Mo(V) without $^1$H splittings.
On applying a high dose of $\gamma$-radiation (ca. 13.5 Mrad.), the relative amount of the Very Rapid Mo(V) increased and so also the $95^{\text{Mo}}$ hyperfine splittings. The spectrum obtained, Fig. 7.7, shows a higher Mo(V) to Fe/S(I) ratio and the $95^{\text{Mo}}$ hyperfine splittings overlapping the Fe/S(I) signals. A dose-dependence of relative amounts of Very Rapid Mo(V) and Fe/S(I) is presented in Section 7.3.3. A stick diagram from the data of Bray and Vanngard (1969) shows a close fitting of the spectrum.

7.3.2 The Fe/S(II) Centre
Spectral characteristics of reduced Fe/S(II) were recorded in the temperature range below 40 K. Identification of Fe/S(II) features proved difficult because of the predominance of OH' ($g_1 = 2.059$, $g_2 = 2.009$, $g_3 = 2.003$), which did not only conceal most of the central features but also, in that temperature range undergoes a temperature-dependent reversible proton loss as earlier reported by Symons, 1982;

$$\text{OH}' + H_2O \rightleftharpoons O'\cdot^- + H_3O^+$$

In frozen aqueous matrix $O'\cdot^-$ has an axial spectrum with g-values of 2.083, 2.083, and 2.002. These positions are close enough to those expected for reduced Fe/S(II) (2.120, 2.007, and 1.910) for them to be mistaken for one another. Samples initially irradiated at 77 K and annealed to ca. 240 K were recooled to various temperatures between 10 and 40 K in order to eliminate both OH' and $O'\cdot^-$. The spectrum obtained at 10 K is shown in Figure 7.8.
Figure 7.6
Comparison of spectra for H$_2$O and D$_2$O samples of xanthine oxidase showing differences in the Rapid Mo(V) signals
Figure 7.7
Spectrum of xanthine oxidase after irradiation with a high dose of γ-rays (ca. 13.5 Mrad.), showing $^{95}\text{Mo}$ splitting on the reduced Fe/S(I) signal.
Figure 7.8
Spectrum of $\gamma$-irradiated xanthine oxidase (at 77K), after annealing to ca. 240K and recooled to ca. 10K, showing features assigned to Fe/S(II)
The features at \( g = 2.112 \) and \( g = 1.903 \) are the \( g_1 \) and \( g_3 \) for Fe/S(II). The \( g_2 (2.007) \) feature is concealed by the central features comprising FADH\(^+\) and protein radical cations. Some amount of Fe/S(I) can be seen to be present under this condition by the \( g = 1.935 \) signal, the \( g_2 \) of Fe/S(I). The broad signal in the high field region in Fig. 7.8 (better resolved in Fig. 7.9) contains \( g_3 \) features for both Fe/S(I) and Fe/S(II) at \( g = 1.903 \) and 1.904 respectively.

The spectrum at 10 K of the unannealed sample, Fig. 7.9, presents evidence for the simultaneous reduction of the two iron centres. Their \( g_3 \) features are distinctly identifiable at 1.896 and 1.904 respectively. The \( g_1 \) and \( g_2 \) for Fe/S(I) are at 2.044 and 1.935 as shown. The \( g_1 \) for Fe/S(II) is at 2.120 while the \( g_2 \) concealed at about free spin is estimated to be 2.007 as earlier obtained by Symons and Petersen (1978).

### 7.3.3 Dose dependent VR Mo(V) and Fe/S(I)

The variation of signal intensities for the Very Rapid Mo(V) and the reduced Fe/S(I) with dose of \( \gamma \)-radiation shows that the molybdenum centre increases in a sigmoid pattern (Figure 7.10a), while the iron centre follows a rather hyperbolic curve (Figure 7.10b). These suggest that electron capture events at the two centres are not identical even if they are simultaneous in occurrence.
Figure 7.9
Spectrum of γ-irradiated xanthine oxidase (at 77K), recooled to ca. 10K
Figure 7.10(a)
Variation of signal intensity of Very Rapid Mo(V) with time of irradiation. Closed triangles are the experimental points, and open triangles are the calculated points.

Time/h

Figure 7.10(b)
Variation of signal intensity of Fe/S(I) with time of irradiation. Closed circles are the experimental points, and open circles are the calculated points.

Time/h
The reduction of xanthine oxidase by γ-radiation at 77 K is considered as a two-step single electron addition comprising two consecutive first order reactions of the type:

\[ A \rightarrow B \rightarrow C \]

\[ \text{XO}_0 \quad \text{XO}_1 \quad \text{XO}_2 \]

\( \text{XO}_0 \) is the fully reduced enzyme which captures one electron at its most electron affinic site Fe/S(I) to produce \( \text{XO}_1 \). The one-electron reduced enzyme is further reduced by the capture of a second electron to give \( \text{XO}_2 \) in which both the Fe/S(I) and the Very Rapid Mo(V) are present.

Since the radiation phenomenon supplies a continuous flux of electrons to the catalytic sites in the protein, the consecutive reactions are treated as decay processes with rate constants \( K_a \) and \( K_b \):

\[ A \xrightarrow{K_a} B \xrightarrow{K_b} C \]

\[ \frac{d[A]}{dt} = -K_a[A] \quad \ldots \ldots \ldots \quad [1] \]

\[ \frac{d[B]}{dt} = K_a[A] - K_b[B] \quad \ldots \ldots \ldots \quad [2] \]

\[ \frac{d[C]}{dt} = K_b[B] \quad \ldots \ldots \ldots \quad [3] \]

From eqn. [1],

\[ \frac{dA}{dt} = -K_a A \quad dt \]

\[ A_t = A_0 e^{-K_a t} \quad \ldots \ldots \ldots \quad [4] \]

Substituting for \( A_t \) in eqn. [2],

\[ \frac{d[B]}{dt} = K_a A_0 e^{-K_a t} - K_b[B] \quad \ldots \ldots \quad [5] \]

Assume the electron capture rates for all reducible sites are the same at 77 K in a constant flux system,

\[ K_a = K_b = K, \]

-151-
This is a first order linear differential equation of the type
\[ B' + KB = L; \quad L = f(t), \]
whose solution is given by
\[ B = e^{-\int K dt} \int L e^{K dt} dt + Je^{-\int K dt} \]
or \[ B = K^{-1} \int L K dt + JK^{-1} \]
where \( K = e^{\int K dt} \), and \( J \) is an integration constant
\[ \therefore B = e^{-Kt} \int K A_0 e^{-Kt} e^{Kt} dt + Je^{-Kt} \quad \ldots \ldots [7] \]
\[ = e^{-Kt} \int K A_0 dt + Je^{-Kt} \]
\[ = e^{-Kt} K A_0 t + Je^{-Kt} \]
\[ \therefore B_t = e^{-Kt} K A_0 t + J(e^{-Kt-1}) \]
At \( t=0 \), \( B=0 \), and hence \( J=0 \)
\[ B_t = K A_0 e^{-Kt} \quad \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots [8] \]
At stationary conditions,
\[ C_t = A_0 - (A_1 + B_t) \]
Substituting for \( A_t \) and \( B_t \) from eqn. [4] and [8],
\[ C_t = A_0 - A_0 e^{-Kt} - K A_0 e^{-Kt} \]
\[ C_t = A_0 [1 - e^{-Kt}(1+Kt)] \]

Relationship to experimental data
Reduced Fe/S(I) arises from \( B \) and \( C \) (\( XO_1 \) and \( XO_2 \)) forms of xanthine oxidase. The growth in Fe/S(I) signal corresponds to the decrease in total enzyme, \( A_t \). The Very Rapid Mo(V) is present in the \( C \) (\( XO_2 \)) form of xanthine oxidase, and
reaches a maximum only after all of the Fe/S(I) centres have been reduced.

Plots of calculated and experimentally determined intensities as functions of electron flux (γ-radiation) are shown in Figure 7.10(a) and 7.10(b). Though the experimental determinations are not kinetic runs in themselves, the use of radiation time as 'reaction time' provides a reasonable fit for the data.

7.3.4 Deductions from analysis
The consecutive reaction mechanism implies that within the enzyme molecule, electrons first migrate to the Fe/S(I) centre via the Mo(VI) centre, acting as a link between the electron-loss centre in the protein moiety and the Fe/S(I) site. Only after the Fe/S(I) is fully reduced do electrons remain at the molybdenum site. This is apparent from the initial rapid rise in the Fe/S(I) signal as against the latent increase in intensity of the Very Rapid Mo(V) signal. The two curves level off at infinite radiation but that of Fe/S(I) approaches a maximum before the Very Rapid Mo(V) curve.
DISCUSSION

7.4.1 Electron-Capture
Most of the previous studies on electron transfer in xanthine oxidase employed chemical reducing substrates like xanthine, sodium dithionite and others for the reduction of the enzyme. The first exception to this method was the work of Symons and Petersen (1978) in which $^{60}$Co γ-radiation was used as the reducing substrate. By this technique, electrons ejected from the protein are captured by different reducible centres in the enzyme. Radical detection was by ESR. Later radiolytic studies were by Battacharyya et al. (1983) and Anderson et al. (1986) in which they used fast spectrophotometry as the monitoring technique. The work reported in this thesis presents further evidence for the reduction of all sites participating in enzymic catalysis and the intramolecular electron transfer reactions between them.

The results show that exposure of frozen aqueous solution of xanthine oxidase to γ-radiation produces the Very Rapid Mo(V), reduced Fe/S(I), reduced Fe/S(II), and the flavin. A comparison of g-values with those in the literature, Table 1, shows that the radiation-reduced centres have ESR parameters identical with those obtained from chemically-reduced species. This proves that dry electrons are sufficiently powerful reducing substrates for initiating enzymic reactions and hence, a useful technique for
studying their catalytic mechanisms.

The primary sites of electron addition are the Mo(VI) and the iron-sulphur centres. Though the sites appear to be competitive, there is remarkable selectivity, being generally more favourable for the Fe/S(I).

It is interesting to note that the present study shows the simultaneous reduction of both the two iron-sulphur centres whereas the earlier similar study did not include this observation. The reducibility of Fe/S(II) by γ-radiation is not unexpected based on the series of investigations by Symons and coworkers on methaemoglobin Fe(III) → Fe(II) (1978a); hemocyanin Cu(II) → Cu(I) (1978b); and manganese complexes Mn(III) → Mn(II) (1982). The present result is in accord with the general phenomenon of one-electron capture by reducible metal centres during γ-irradiation of metalloproteins. The degree of reduction however would vary depending on their reduction potentials. Though G-values have not been quantified in this study, signal intensities of ESR spectra provide a basis for comparison of relative reduction potentials or electron affinities at 77 K.

7.4.2 The Very Rapid Mo(V) and the Rapid Mo(V)

The transition from Very Rapid to Rapid Mo(V) takes place most significantly between 150 and 185 K, though some Rapid may have been formed at 77 K, (see Figure 7.11).
Figure 7.11
Spectra of γ-irradiated xanthine oxidase (at 77K) annealed to various temperatures, showing loss of the Very Rapid Mo(V) signal and the growth of the Rapid Mo(V) without any change in the Fe/S(I) signal.
This transition is accompanied by the protonation of a site close to the molybdenum centre as already reported in the literature. The temperature range however is not often mentioned but it is particularly noteworthy for two reasons. The first is the unique occurrence of the Very Rapid for the xanthine-reduced enzyme (followed by rapid freezing) as against its non-occurrence with other chemical reducing substrates. The second reason is the complete disappearance of the molybdenum Very Rapid signal as a precursor to the formation of the Rapid species.

In the irradiation of ices (at 77 K) OH' radicals produced undergo the reaction

$$\text{OH}' + \text{OH}' \rightarrow \text{H}_2\text{O}_2$$

at ca. 130 K. This is a bimolecular reaction. At this temperature other reactions occurring by intermolecular collision would be expected to commence, leading to various additional products. The protonation of Very Rapid Mo(V) does not occur until ca.150 K a temperature well above that at which OH' decays. It may be inferred that the Very Rapid Mo(V) undergoes a structural relaxation (at ca.150 K) during which the specific site becomes available for protonation in order to form the Rapid species. In the case of the xanthine-reduced enzyme, this relaxation is probably inhibited through binding of (xanthine)$^+$ at such a site that protonation does not commence before rapid freezing. As for other chemical reducing substrates, substrate binding to enzyme may be so slow that relaxation of reduced enzyme would have taken place and protonation already commenced before rapid freezing quenches the
reaction. An alternative explanation may be that the other chemical reducing substrate for an ES complex is structurally different from the xanthine-enzyme complex at least in the environment of the protonation site.

The spectra in Fig.7.11 show a gradual decay of the Very Rapid Mo(V) until it virtually disappears at ca. 150 K, being replaced at ca 185 K by the Rapid. The loss of Mo(V) is attributed to either reoxidation to Mo(VI) or further reduction to Mo(IV), both of which are ESR silent. The latter is thought to be the case; the electron having been gained from one of the iron-sulphur centres, most likely Fe/S(I). This explanation is supported by the loss of reduced Fe/S(I) signal during the annealing. In the unlikely event that Very Rapid Mo(V) was reoxidised to Mo(VI), electrons lost from the molybdenum and Fe/S(I) centres would pass on to Fe/S(II) and the flavin. That this is not the case is proven from comparative redox potentials obtained by Olson et al (1974). Furthermore, a reversal to Mo(VI) must have a positive ΔG (since the initial reduction was spontaneous; -ve ΔG), hence a thermodynamically unfavourable event. One other point is the fact that for Rapid Mo(V) to emerge from Mo(VI), an electron-capture event is inevitable.

The occurrence of Mo(IV) in the scheme of enzymatic mechanism had earlier been suggested by Bray and George (1985) but then it was only as a precursor to the Very Rapid Mo(V). In this discussion it is being proposed as an intermediate between the Very Rapid and the Rapid Mo(V) species. Reduction to the +4 oxidation state is an aspect
of the structural relaxation mentioned above. It is noted also that the disappearance of the Very Rapid occurs through a faster decay of the $g_3$ feature than the $g_2$ in the spectrum in Fig.7.11. This is thought to be part of the structural relaxation of the molybdenum centre during the transition.

7.4.3 $D_2O$ Sample

Fig.7.12 shows the spectra obtained for the Rapid Mo(V) in the normal enzyme and its deuterated analogue. The proton splitting observed in the normal enzyme is found to be absent in the deuterated sample. The $g_2$ and $g_3$ doublet features have been replaced by singlets. This is a reaffirmation of earlier results, especially those obtained with chemical substrates.

CONCLUSION

$\gamma$-irradiation of xanthine oxidase results in the selective capture of electrons by the reactive sites of the enzyme to produce partly reduced enzyme species. The reactive intermediates have ESR parameters identical with those obtained using chemical reducing substrates.

Electron capture at the Fe/S(I) centre is faster than at all other electron-affinic sites. Reduction of Mo(VI) is preceded by the complete reduction of Fe/S(I) for which it acts as an electron transfer intermediate; a case of two consecutive electron-transfer events.
REFERENCES


