REACTIONS AND LIGAND BINDING PROPERTIES
OF CYTOCHROME P450

A thesis presented for the Degree of Doctor of Philosophy

by

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ABSTRACT.

One of the most interesting and characteristic reactions catalysed by cytochrome P450 is the hydroxylation of alkanes and unactivated alkyl groups (\(O_2 + 2H + RH \rightarrow ROH + H_2O\)), which is always accompanied by some degree of "uncoupling" to produce hydrogen peroxide (\(O_2 + 2H \rightarrow H_2O_2\)); added hydrogen peroxide can also be used by some cytochrome P450 enzymes for the hydroxylation of alkanes (\(H_2O_2 + RH \rightarrow ROH + H_2O\)). Major unanswered questions concerning these reactions include: the nature of the key hydroxylating intermediate and the possible role of the two conformers observed in the reduced state with ligands such as isocyanides and pyridine (corresponding to Soret bands at 440-450 and 420-430 nm). These questions were investigated by studying (i) the reactions of oxidised CYP2B4 with \(H_2O_2\) and lauric acid and (ii) equilibria involving reduced CYP2B4 with a range of nitrogen bases.

It was shown that purified CYP2B4 catalysed the hydroxylation of lauric acid by hydrogen peroxide producing the \(\omega-1\) and \(\omega-2\) hydroxymetabolites against a background of bleaching of the porphyrin ring with no detectable intermediate in either reaction. Iodosobenzene on the other hand, which is known to produce the ferryl FeO\(^{3+}\) derivative, does not support hydroxylation or bleaching and further inhibits bleaching by added hydrogen peroxide and that the rates of both bleaching and hydroxylation increase with the concentration of \(H_2O_2\) (pK 11.7) but are essentially pH-independent. The available evidence indicates that the key intermediate in both hydroxylation and bleaching is the undissociated hydrogen peroxide (\(H_2O_2^*\)) coordinated to the Fe\(^{3+}\) ion.

The nature of the equilibrium between conformers I and II, reported for complexes of reduced cytochrome P450, was further investigated through the use of three families of nitrogenous bases in order to test the relative
importance of σ donor and π acceptor capacity and the α effect. Equilibrium was systematically shifted in favour of II by increasing the pK in all three families, indicating the predominance of σ donor over π acceptor capacity, as well as by lowering the pH. This is in agreement with the postulated equilibrium between thiol/thiolate as the proximal ligand in conformers II and I respectively. Ligands such as hydroxylamine and pyridazine, which exhibit the α effect were anomalous in showing an unexpectedly high stabilisation of conformer II, which was observed with CYP4A1 and CYP2E1 as well as CYP2B4 and provides a model for the similar anomalous behaviour of oxygen as a ligand.
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# CONTENTS

| Title page | i |
| Abstract | ii |
| Acknowledgements | iv |
| Contents | v |

## Chapter 1.0 General Introduction
1.1 Cytochrome P450 nomenclature  
1.2 Multiplicity of cytochrome P450  
1.3 Active site  
1.4 Reactions of cytochrome P450  
1.5 Reactions with other oxygen donors  
1.6 Nature of reactive intermediate  
1.7 Nature of the sixth ligand in the resting state  
1.8 Binding of ligands to ferric cytochrome P450  
1.9 Binding of ligands to ferrous cytochrome P450  
1.10 Thromboxane synthase and allene oxide synthase  
1.11 Cytochrome P420  
1.12 Chloroperoxidase  
1.13 Haem oxygenase  
1.14 Aims

## Chapter 2.0 Purification and characterisation of CYP2B4
2.1 Introduction  
2.2 Materials  
2.3 Methods  
2.3.1 Preparation of column chromatography media  
2.3.2 Preparation of column apparatus  
2.3.3 Purification of CYP2B4  
2.3.4 DEAE cellulose anion exchange chromatography  
2.3.5 Hydroxylapatite column chromatography  
2.3.6 CM-sepharose column chromatography  
2.3.7 Purification of NADPH cytochrome P450 reductase  
2.3.8 Adenosine 2',5'-diphosphate agarose column chromatography  
2.3.9 Concentration of various protein fractions  
2.3.10 Spectrophotometric enzyme assays  
2.3.11 Characterisation of purified CYP2B4 fraction
2.4 Results
2.4.1 Purification of rabbit CYP2B4 47
2.4.2 Characterisation of pure and partially purified CYP2B4 50
2.5 Discussion 54

Chapter 3.0 Reactions of oxidised CYP2B4 involving hydrogen peroxide 57
3.1 Introduction 58
3.2 Materials 62
3.3 Methods 63
3.3.1 Preparation of partially purified and pure CYP2B4 63
3.3.2 Binding of nitrogenous bases to oxidised CYP2B4 63
3.3.3 Determination of binding constants at ferric level 63
3.3.4 The effect of hydrogen peroxide on CYP2B4 64
3.3.5 The action of iodosobenzene on CYP2B4 65
3.3.6 The action of hydrogen peroxide on lauric acid hydroxylation 66
3.3.7 The effect of various parameters on lauric acid hydroxylation 67
3.3.8 Lauric acid hydroxylation using a reconstituted system 68
3.3.9 Effect of pH on lauric acid hydroxylation 69
3.3.10 Effect of tergitol on lauric acid hydroxylation 69
3.3.11 Effect of iodosobenzene on lauric acid hydroxylation 70
3.4 Results 71
3.4.1 The binding of ligands to oxidised CYP2B4 71
3.4.2 Determination of binding constants of ligands to oxidised CYP2B4 72
3.4.3 Action of hydrogen peroxide on CYP2B4 80
3.4.4 Effect of hydrogen peroxide concentration 81
3.4.5 Effect of substrates on rate of degradation of CYP2B4 82
3.4.6 Hydrogen peroxide supported lauric acid hydroxylation 83
3.4.7 Identification of metabolites of lauric acid hydroxylation 86
3.4.8 Effect of incubation time 87
3.4.9 The products from lauric acid hydroxylation supported by various oxygen donors 90
3.4.10 Effect of hydrogen peroxide concentration 93
3.4.11 Effect of pH on lauric acid hydroxylation 94
3.4.12 Effect of lauric acid concentration 95
3.4.13 Effect of tergitol 97
3.5 Discussion 98
3.6 Conclusion 107
CHAPTER 4.0 Coordination of ligands by the reduced (Fe$^{2+}$) ion.

4.1 Introduction
4.2 Materials
4.3 Methods
  4.3.1 Spectrophotometric assays
  4.3.2 Effect of pH on ligand binding to reduced CYP2B4
  4.3.3 Effect of varying pH on pyridine produced spectrum
  4.3.4 Determination of binding constants at reduced level
  4.3.5 Effect of time and ligand concentration on binding to reduced CYP2B4
  4.3.6 Binding of nitrogen ligands to different families of P450
  4.3.7 Competition between ligands for reduced CYP2B4
  4.3.8 Identification of the ligand involved in ligand binding (EXAFS)
  4.3.9 Investigation into the role of the Thr residue
  4.3.9.1 Preparation of the thromboxane synthase enzyme
  4.3.9.2 Determination of total protein and haem content
  4.3.9.3 Ligand binding to thromboxane synthase
  4.3.10 Ligand binding at the ferric level
  4.3.11 Determination of binding constant at ferric level
  4.3.12 Effect of time and extra ligand on binding at ferric level
4.4 Results
  4.4.1 Effect of pH on binding of nitrogen bases to reduced P450
  4.4.2 Effect of varying pH on difference spectra produced by pyridine
  4.4.3 Determination of relationship between pKa and binding
  4.4.4 Determination of binding constants at reduced level
  4.4.5 Effect of concentration and time
  4.4.6 Binding of ligands to different P450 families
  4.4.7 Competition between ligands for reduced P450
  4.4.8 EXAFS
  4.4.9 Investigation into the role of threonine
  4.4.10 Ligand binding to ferric CYP2B4
  4.4.11 Determination of binding constant at ferric level
4.5 Discussion
4.6 Conclusion
CHAPTER 1

General Introduction
1.1 Cytochrome P450 nomenclature.

The cytochromes P450 (EC 1.14.14.1) comprise a family of b-type haemoproteins (Sato et al. 1965), with an identical prosthetic group (a thiolate-bound haem) and mechanism of catalysis (activation of oxygen without activating the substrates), but widely different apoprotein structures which are responsible for their different substrate specificities. The term cytochrome P450 actually refers to a group of haemoproteins, which includes the enzyme chloroperoxidase whose Fe²⁺-carbon monoxide complexes show an absorption spectrum with a maximum near 450 nm. This characteristic is attributed to the unique ligation of the haem by a cysteinyl thiolate (Guengerich 1992). The cytochrome P450 enzymes are mainly classed as monooxygenases or mixed function oxidases, i.e. they are oxygenases which catalyse the incorporation of only one atom of molecular oxygen into another substrate and yield a molecule of water with the other oxygen atom (equation 1) (Schenkman et al. 1981), where RH represents the substrate and ROH the product.

\[
RH + O_2 + 2H \rightarrow ROH + H_2O
\]  

(1)

Cytochrome P450 may be divided into 2 classes, depending on the nature of their redox partner. Class I cytochrome P450s are found in the mitochondrial membranes of eukaryotes and in most bacteria and require a flavin adenine dinucleotide (FAD) containing reductase and an iron-sulphur protein for catalysis. Class II cytochrome P450s, on the other hand, are located on the endoplasmic reticulum and interact directly with a reductase moiety containing FAD and flavin mononucleotide (FMN) (Boddupalli et al. 1992). The actual term cytochrome P450 is a misnomer, as these proteins are not "cytochromes" in the true meaning of this terminology. The actual term to describe these proteins, as emphasised by the Nomenclature Committee of the International Union of Biochemistry (NC-IUB) is "haem-thiolate proteins" (Palmer and Reedijk 1991).
The cytochrome P450 enzymes are ubiquitous and are even found in bacteria. According to the most recent update, there are currently 221 P450 genes and 12 putative pseudogenes which have been described in 31 eukaryotes (including 11 mammalian and 3 plant species) and in 11 prokaryotes (Nelson et al 1993). Of the 36 gene families so far identified, 12 exist in all mammals examined to date, and comprise 22 mammalian subfamilies. Cytochrome P450s are considered to be in the same family if they share greater than a 40% sequence homology, whilst subfamilies are constituted by cytochrome P450s which share at least 60% sequence homology. Cytochrome P450's are named with the root symbol "CYP" followed by an arabic number designating the P450 family. A letter is used to indicate the subfamily when two or more subfamilies are known to exist within the family, and an arabic number to represent the individual gene.

1.2 Multiplicity of cytochrome P450.

In the untreated animal, levels of the P450 enzymes are very low; they comprise approximately 5% of the total hepatic microsomal protein content. The levels of these enzymes can be increased dramatically by treatment with chemical inducers such as phenobarbital and 3-methylcholanthrene. The induction of cytochrome P450 by the administration of phenobarbital and related compounds is generally more pronounced in the liver than in nonhepatic tissues. In the rat, rabbit and mouse, treatment with phenobarbital causes a significant increase in total hepatic cytochrome P450 concentration, proliferation of smooth endoplasmic reticulum, and increased liver weight (Remmer et al 1983). The major inducible subfamilies of cytochrome P450 are CYP1A, CYP2B, CYP2E, CYP3A and CYP4A. The CYP2 family is recognised as the largest and most diverse of the cytochrome P450 families. To date, the CYP2 family consists of eight subfamilies (Nelson et al 1993), with the CYP2B gene subfamily the largest with at least 10 members (Gonzalez 1990; Nebert et al 1989). Phenobarbital-type inducers are known to regulate levels of the various CYP2 isoforms and
can increase by 15 - 40 % the amount of enzyme present. At least five different forms of cytochrome P450 have been purified from phenobarbital-induced rabbits, the major inducible form being CYP2B4, which has been characterised by many laboratories (Dean and Coon 1977; Guengerich 1977; Haugen and Coon 1976). The molecular weight of the CYP2B4 subunit has been calculated to range from 48,500 to 50,000, with an absorption maximum of 451 nm for the reduced-carbon monoxide complex. The dramatic increase in the amount of enzyme present on induction by phenobarbital and the large number of purification methods available advocate the use of this enzyme in future experiments.

1.3 The Active site.

The active site of all cytochrome P450s contains an iron (III) protoporphyrin IX, where the iron is linked to the apoprotein by a cysteine residue as the proximal ligand. To date, the structure of three bacterial cytochrome P450s (P450cam, P450BM3 and P450terp), have been elucidated using X-ray studies. Initial studies on the active site of cytochrome P450 were carried out on P450cam, a class I soluble enzyme from *Pseudomonas putida* and has yielded the most complete 3D information on the cytochrome P450 active site (Poulos and Raag 1992; Poulos *et al* 1987). The crystal structure of P450cam has provided guidelines for understanding the stereospecificity of the hydroxylation reaction, changes in redox potential, and spin equilibria on substrate binding. However, the lack of definitive amino acid sequence similarity between P450cam and eukaryotic P450s limits its usefulness as a general model for all cytochrome P450s (Poulos 1992). The only well characterised class II bacterial P450 is P450BM-3 from *Bacillus megaterium*, which is involved in the monooxygenation of fatty acids producing the ω-1, ω-2 and ω-3 hydroxymetabolites (Fulco 1991). Cytochrome P450BM-3 and P450cam both possess conserved residues (threonine
and glutamine) in the binding site, which are identical to other cytochrome P450s. The conserved threonine in P450BM-3 may play an important role in proton transfer via a charge relay mechanism (Gerber and Sligar 1992). Comparison of the active site of cytochromes P450terp and P450cam shows that both active sites contain a conserved threonine residue, which is hydrogen bonded to carbonyl oxygen, and an aspartate residue which is -1 base from the threonine residue which links surface residues and/or solvent molecule to the catalytic centre of both proteins (Hasemann et al 1994). So far, no mammalian active site has been completely described, although computer modelling has proposed that homology does exist between P450cam and mammalian P450s. However this is limited to a single 8 residue region (Schwarse et al 1988) which is proposed to contain important residues including threonine, serine, and aspartate which are all involved in the binding of oxygen. The nature of the proximal (fifth) ligand in all cytochrome P450s is known to be a Cys residue in its anionic RS²⁻ form (Cramer et al 1978). However, it is possible that an equilibrium exists between this Cys residue in its RS⁻ (thiolate) or RSH (thiol) form indicated by the following equilibrium:-

\[
[\text{RS}^- \Leftrightarrow \text{Fe(II) \Leftrightarrow X}_1 + \text{H}^+ \Leftrightarrow [\text{RSH}^\bullet \Rightarrow \text{Fe(II) \Leftrightarrow X}_2]
\]

where X denotes the distal ligand and subscripts I and II the two conformers; H⁺ could represent either a free proton in solution or a proton transferred from some neighbouring site (Kahl et al 1976). Some of the other enzyme systems classed as P450s, namely thromboxane synthase and allene oxide synthase lack the threonine and serine residues in the active site and are involved in reactions which do not involve oxygen, thus suggesting that these residues are important for the binding of oxygen.
1.4 Reactions of cytochrome P450.

The substrates metabolised by cytochrome P450 include a whole host of xenobiotics, synthetic organic chemicals, steroids and physiological lipids (Black and Coon 1987; Ryan and Levin 1990; Guengerich 1991; Coon and Koop 1983). The reactions catalysed by cytochrome P450 include hydroxylation (aromatic and aliphatic), epoxidation, deamination (amphetamine $\Rightarrow$ phenylacetone and ammonia), dehalogenation, dealkylation and reduction as well as peroxidase type reactions (equation 2), where $\text{AH}_2$ is the reducing substrate.

$$\text{ROOH} + \text{AH}_2 \Rightarrow \text{ROH} + \text{A} + \text{H}_2\text{O} \quad (2)$$

The initial stage of the cytochrome P450 catalysed monooxygenase reaction involves the transfer of electrons from NAD(P)H to either NADPH-cytochrome P450 reductase in the microsomal system or a ferrodoxin reductase and a nonhaem iron protein in the mitochondrial and bacterial systems, followed by transfer to the Fe$^{3+}$ ion of cytochrome P450. This subsequently leads to the splitting of the dioxygen bond followed by the insertion of one oxygen into the substrate. Hydroxylation reactions are known to be carried out with the following stoichiometry, where RH is the substrate and ROH is the product:

$$\text{RH} + \text{O}_2 + \text{NADPH} + \text{H}^+ \Rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}^+$$

Our interest in this reaction is when RH represents a simple alkane or some equivalent unactivated alkyl group as in the more soluble lauric acid. Not all alkanes can be hydroxylated by cytochrome P450, for example methane. The hydroxylation of methane is catalysed by the enzyme, methane monooxygenase (MMO), which like cytochrome P450 can utilise oxygen, NADPH, and a reductase or hydrogen peroxide on its own (Froland et al 1992). Controversy still surrounds the nature of the key intermediate involved in the hydroxylation reaction. Two main theories have been proposed for the nature of the key intermediate involved in the hydroxylation reaction viz (1) a single oxygen unit (Guengerich 1990), or (2) a two oxygen (peroxide) unit (White and Coon...
1980). The most popular theory is the one involving a single oxygen unit in the form of a ferryl intermediate \((\text{FeO})^{3+}\). A postulated mechanism for microsomal cytochrome P450 catalysis is depicted in figure 1.1.

Fig 1.1 Proposed scheme for the mechanism of action of cytochrome P450. Fe represents the haem iron atom in the active site, RH a substrate, and ROH the corresponding monooxygenation product. XOOH represents a peroxy compound that serves as a oxygen donor. (adapted from Coon et al 1992).
Although the various steps of this cycle have been examined in some detail, only steps 1-3 are well understood with controversy still surrounding steps 4-7. The binding of the substrate to the ferric form of cytochrome P450 is thought to be very rapid and is the first stage of the cycle (step 1). The binding of the substrate changes the iron spin-state (d electrons) from low spin to high spin in some cases, but not in others (where the iron moiety already exists in the high spin state without substrate binding) (Guengerich 1983). Step 2 involves the first electron reduction of the substrate-bound ferric cytochrome P450 to the ferrous form of the haemoprotein. The reducing equivalent in the microsomal system is derived from NADPH and H+ and is transferred by the flavoprotein, NADPH cytochrome P450 reductase. This is followed by the binding of molecular oxygen to the ferrous cytochrome P450 substrate adduct and is thought to be rapid due to the (Fe(II)O2) complex being very unstable (step 3). Steps (4-7) are not well understood, but are thought to involve transfer of a second electron either from NADPH cytochrome P450 reductase or b5, to generate an activated iron-oxygen complex (step 4) (Schenkman 1976). This is followed by the splitting of the O-O bond with the uptake of 2 protons and the generation of an "activated oxygen" species, with the simultaneous release of a water molecule (step 5). Oxygen is then incorporated into the substrate (steps 6 and 7). Finally, the oxygenated product dissociates (step 8) to leave the iron in its original ferric state.

The overall hydroxylation reaction (equation 1) is always accompanied by the production of hydrogen peroxide via reaction 3, which eventually leads to the degradation of the porphyrin ring.

\[
O_2 + 2H \rightarrow H_2O_2 \quad (3)
\]

The production of hydrogen peroxide is enhanced in the presence of uncouplers such as C6F12 (Hawkins and Dawson 1982). This degradation of the porphyrin ring of cytochrome P450 by hydrogen peroxide has similar parallels to the
degradation of haem oxygenase by hydrogen peroxide. Haem oxygenase, which contains histidine (His) as its proximal ligand functions to degrade haemin and requires either NADPH, molecular oxygen and a reductase, or hydrogen peroxide on its own. Cytochrome P450 can also catalyse the reaction of hydrogen peroxide with alkanes (RH) (equation 4).

\[
\text{RH} + \text{H}_2\text{O}_2 \rightarrow \text{ROH} + \text{H}_2\text{O} \quad (4)
\]

The occurrence of (3) and (4), involving the formation and further reaction of hydrogen peroxide can in principle allow for the study of the overall reaction (1) in two segments, and investigation into the nature of the key intermediate and the possible role of the reduced iron conformers in the enzymic cycle.

1.5 Reactions with other oxygen donors.

Cytochrome P450 enzymes are usually activated by the sequential addition of an electron (via NADPH-cytochrome P450 reductase), molecular oxygen and another electron followed by protonation and loss of water. However, this process can be circumvented by the use of artificial oxidants, such as hydroperoxides (cumene hydroperoxide and hydrogen peroxide) (Kadlubar et al 1973), iodosobenzene (Lichenberger et al 1976), sodium periodate and sodium chlorite (Gustafsson and Bergman 1976) leading to a shortened P450 cycle. It is generally assumed that this shortened cycle or peroxide shunt proceeds via a common intermediate, bearing a single activated oxygen atom, which is similar to compound I of the peroxidase enzyme (Sligar et al 1977). However, this view is not supported by all workers in this field (White and Coon 1980). Hydrogen peroxide supports the peroxygenase activity of cytochrome P450 less effectively than cumene hydroperoxide with some substrates (Kadlubar et al 1973; Nordblom et al 1976) but not with others (Vaz et al 1991). Furthermore hydrogen peroxide is a very poor supporter of the peroxidase activity (Hrycay and O'Brien 1974). The most useful oxygen donor has been found to be
iodosobenzene, a lipophilic agent that readily interacts with the membrane bound enzymes, it is a single oxygen donor and is uncharged unlike IO$_4^-$ and ClO$_2^-$. The rates of oxidation by these artificial donors has been found to be much higher than those observed with NADPH, reductase and molecular oxygen (Macdonald et al 1989).

1.6 Nature of the reactive intermediate.

Initially it was assumed that the intermediate in these reactions, especially in the case of iodosobenzene was the same as for peroxidases, namely FeO$_3^{3+}$; due to the observation of spectra of the intermediate complexes when iodosobenzene was added to a sample of purified cytochrome P450 (Blake and Coon 1989). The cleavage of the O-O bond in the catalytic cycle is extremely rapid and has never been observed. The nature of the intermediate formed after this cleavage is open to speculation. It is thought that the intermediate consists either of a single oxygen unit, termed the ferryl intermediate (FeO)$_3^{3+}$, or a two oxygen unit (FeO$_2$H/H$_2$O$_2$), although neither has been conclusively proved or disproved.

Both these theories concerning the key intermediate seem reasonable since it has been shown that a simple protonated hydrogen peroxide molecule (H$_3$O$_2^+$) can hydroxylate alkanes (Olah et al 1977). The hydroxylation of these alkanes with hydrogen peroxide in the presence of strong acids such as FSO$_3$H-SbF$_3$, FSO$_3$H, H$_2$SO$_4$ and HF was studied under typical electrophilic conditions, where it was concluded that the reactions proceeded via initial electrophilic hydroxylation of the appropriate α bond of the alkanes by the incipient hydroxyl cation which is formed through protolytic cleavage of hydrogen peroxide through the hydroperoxonium ion H$_3$O$_2^+$. It is therefore reasonable to assume that hydrogen peroxide attached to another Lewis acid (eg. Fe$_3^{3+}$) might be able to also hydroxylate alkanes. It is possible that the key intermediate could exist as
both forms, but used under different circumstances.

The reaction of cytochrome P450 with iodosobenzene has been shown to produce the ferryl intermediate, although this intermediate is never seen during the catalytic reaction (Blake and Coon 1989). The use of iodosobenzene as the oxygen donor in the absence of molecular oxygen and NADPH, but in the presence of cytochrome P450 catalyses the oxygen transfer reaction:

\[ \text{RH} + \text{PhIO} \rightarrow \text{ROH} + \text{Phi} \] (5)

where RH represents a variety of substrates and PhIO a variety of iodosobenzene derivatives that serve as oxygen donors, and neither molecular oxygen nor an external electron source is required (Nordblom et al 1976). The reaction of CYP2B4 with iodosobenzene has previously been shown to lead to the reversible formation of three spectral intermediates, termed E, F and G, where E represents the iodosobenzene-dependent partial shift of the low spin 6-coordinate form of the ferric enzyme to the high spin 5-coordinate form. Complex F represents a transient intermediate, whose spectrum cannot be determined, whilst G represents a blue shifted (shorter wavelength) intermediate with an absorption maximum at \( \sim 390 \text{ nm} \) in the absolute spectrum. The spectrum of complex G has been found not to vary when different derivatives of iodosobenzene were used, which is in contrast to the reactions of peroxy compounds (cumene hydroperoxide) with CYP2B4 (Blake and Coon 1989). Complex G was found to exhibit spectral properties which maybe associated with an iron-oxo intermediate \((\text{FeO})^{3+}\), which contains one oxygen derived from the starting compound (resembles compound I of peroxidases), with a blue shifted Soret band of less intensity.

Recent studies carried out using distal ligand mutants of CYP1A2, where the Glu ligand was replaced by either Ala, Val, Gln or Asp, and the Thr ligand replaced by either Ala, Gln, Val, Asp, or Ser (Shimizu et al 1994) suggested
the formation of a ferryl intermediate which can be observed spectrally at a wavelength of \(\sim 423\) nm, when butyl hydroperoxide and cumene hydroperoxide were used as the oxygen donor. The intermediate observed at this wavelength was proposed to be similar to that of compound I of the peroxidases. However, these experiments were carried out in the presence of 1 mM dithiothreitol, which would have most certainly reduced the intermediate formed. Thus, due to the presence of this thiol no conclusive proof can be drawn from these experiments.

The two oxygen theory, although not currently the most popular, is gaining support. The presence of a two oxygen unit intermediate has been proposed to be involved in the reaction involving cytochrome P450 and cyclohexane carboxyaldehyde which yields formic acid and cyclohexene as the products

\[
(R\text{.CHO} + H_2O_2 \Rightarrow (R\text{-H}) + \text{HCOOH} + H_2O) \ (Vaz \text{ et al } 1991).
\]

This reaction was found to be dependent on NADPH, molecular oxygen and a reductase in a reconstituted system; in the absence of these reducing equivalents, only hydrogen peroxide and not other oxidants such as iodosobenzene, m-chloroperoxidase and cumene hydroperoxide could initiate the reaction. The inability of iodosobenzene to support the reaction involving cyclohexane carboxyaldehyde and cytochrome P450 suggested that the pentavalent "iron oxene" was not the key intermediate and that a peroxide type intermediate may be involved. Further support for the existence of a peroxide type intermediate has been proposed by workers involved in the study of haem oxygenase, which destroys haemin (Wilks and Ortiz de Montellano 1993; Wilks et al 1994) and studies involving cytochrome P450_{17a} (Akhtar et al 1994; Robichaud et al 1994). The reactions involving haem oxygenase will be expanded later (see 1:13), but the general consensus is that the degradation of haemin by haem oxygenase does not involve a ferryl intermediate but proceeds via a two oxygen unit.
Cytochrome P450\textsubscript{17\alpha} is involved in steroid biosynthesis as well as in acyl-cleavage for which the participation of an iron peroxide intermediate (FeOOH) has been suggested. It was found that the reaction involving the formation of 17\alpha hydroxyandrogen from the C-C bond cleavage process did not go via a ferryl intermediate, unlike the reaction with other steroid enzymes such as 14\alpha-demethylase, but did proceed via some peroxide intermediate. The proposed existence of this peroxide species, although it has never been seen and the inability of iodosobenzene to support the cyclohexane carboxyaldehyde reaction suggests that reactions involving hydrogen peroxide involve a two rather than one oxygen unit as the key intermediate. The existence of some common features between cytochrome P450 and haem oxygenase provided further motivation to investigate whether a one oxygen or two oxygen unit was involved in the hydroxylation reactions of alkanes.

1.7 Nature of the sixth ligand in the resting state.

In the ferric low spin state, the CYP2B4 haem iron atom is hexacoordinated and contains a solvent molecule which occupies the axial sixth coordination position \textit{trans} to the thiolate fifth ligand (Poulos et al 1987). Controversy surrounds the nature of this solvent molecule as the sixth ligand; some authors advocate the presence of a hydroxide ion (Banci et al 1994), whilst other propose a water molecule (Hilderbrandt et al 1994).

NMR signals from camphor free cytochrome P450 in the presence of aromatic bases such as pyridine, imidazole and N-methylimidazole showed that the bases bind both in the hydrophobic cavity and at the metal. It is thought the nature, size and steric crowding of the ligand determines the displacement or the presence of the axial solvent ligand and its protonation state (Raag and Poulos 1989). In the presence of the natural substrate, camphor, the iron (III) is
pentacoordinated and is in a high spin state, due to the displacement of the solvent molecule by the substrate molecule. The simultaneous binding of pyridine and imidazole in the cavity was found not to occur in the presence of the substrate.

The existence of a water molecule as the sixth ligand has been proposed by Hilderbrandt and co-workers (1994), using Resonance Raman spectroscopy. This technique was used to study mitochondrial cytochrome P450\textsubscript{Scc} and microsomal cytochrome CYP2B4 in the ferric substrate-free and substrate-bound states. The spectra of cytochrome P450\textsubscript{Scc} showed the existence of two conformational states, known as A and B. Each of these states constituted an equilibrium between a six coordinated low spin and a high spin form. Both the conformational and the spin equilibria were found to be pH and temperature dependent (Lange et al 1992). The pH dependency between the low spin and high spin states of cytochrome P450\textsubscript{Scc} suggests that the sixth ligand was a water molecule, and the transition between the 6 coordinated high spin and 6 coordinated low spin states of conformer A is initiated by a proton transfer step. In contrast to cytochrome P450\textsubscript{Scc} the Resonance Raman spectra of microsomal CYP2B4 provided no indications for multiple conformers at a temperature of 22 °C (Hildebrandt et al 1994). The binding of certain substrates (benzphetamine) to ferric CYP2B4 can convert the low spin state to the high spin state, but this conversion is only partial (28 %). The Resonance Raman spectra of this conversion produced similar values to that obtained for the high spin state of P450\textsubscript{Scc}, suggesting that a water molecule is present as the sixth ligand.
1.8 Binding of ligands to ferric cytochrome P450.

As previously stated CYP2B4 exist essentially in the low spin 6-coordinate state with a ferric Soret absorption at around 418 nm. When certain substrates such as carbon tetrachloride and halothane, which are termed type I substrates, bind to low spin (s=1/2) cytochrome P450, they usually bind to the protein part of the molecule and change the conformation, and hence the ligation of the haem prosthetic group to the 5-coordinate high spin (s=5/2) state, although this conversion is not complete. The change from low spin to high spin causes a characteristic spectral change termed the type I spectral change (Schenkman et al 1967), with an absorption maximum at around 390 nm and minimum around 420 nm in the difference spectrum. In contrast to type I substrates, certain substrates such as, nitrogenous bases termed type II substrates are thought to ligate to the haem iron of cytochrome P450, resulting in a 6-coordinated, low spin haemoprotein, which is characterised by an increase in absorbance at 425-435 nm and a trough at 390 - 405 nm (Schenkman et al 1967). The position of the absorbance maximum in the Soret spectrum of the ferric protein is related to the spin state of its iron centre as measured by resonance techniques such as EPR and Mossbauer spectroscopy (Sligar 1976; Lange et al 1980). A characteristic of the low spin ferric state of cytochrome P450 is that the α band is more intense than the β band (Schenkman et al 1967).

1.9 Binding of ligands to ferrous cytochrome P450.

The term cytochrome P450 is derived from the observation that ferrous-CO state of the enzyme displays a Soret absorption maximum at approximately 450 nm. This spectral feature is rather unique among CO-binding protohaem proteins in that all ferrous-CO protohaem systems other than cytochrome P450 and chloroperoxidase absorb at 420 nm. The formation of this band is associated
with the presence of Cys in its anionic form $RS^-$ as the proximal ligand (Guengerich 1991). Unusual features of the ferrous carbon monoxide spectra include coalescence of the normally discrete $\alpha$ and $\beta$ bands into a single feature at 550 nm and the presence of an especially intense $\delta$ transition near 370 nm, which were matched by thiolate-ligated ferrous carbon monoxide haem models (Gaul and Kassner 1988).

The interaction of certain ligands with ferrous cytochrome $P450$ produces complexes with two absorption peaks in the Soret region of the visible spectrum. This type of spectrum was first observed with the isocyanide derivatives which produced peaks at 430 nm and 455 nm (Imai and Sato 1966a-b). The formation of these two peaks was associated with the presence of pH-dependent interconvertible states or conformers (Imai and Sato 1967a) which were later denoted II and I, with conformer I (455 peak) favoured by higher pH (Tsubaki et al 1989). The equilibrium between I and II may vary between isozymes (Sladek and Mannering 1966); with the ratio between the two conformers varying depending on animal pretreatment. Pretreatment of animals with phenobarbital produced peaks at 430 and 455 nm, whilst pretreatment with 3-methylcholanthrene produced peaks at 430 and 453 nm. Equilibrium between the two conformers was also seen between wild types and mutants (Kraniev et al 1991), where the peaks were at 451 and 423 nm (conformer I and II respectively), and is displaced in favour of II by a conformational change induced by the binding of adrenodoxin to the mitochondrial P450$_{bc}$ (Tsubaki et al 1989). However, in the bacterial P450$_{cam}$, the presence of substrate (camphor) is known to impose greater rigidity on the active site and appears to stabilise conformer I and/or inhibit any pH-dependent equilibrium (Greschner et al 1993). This type of double spectrum can also be produced with compounds such as pyridine (Imai and Sato 1967a), aniline (Imai and Sato 1967a-b) and phosphites (Dahl and Hodgson 1976a).
The binding of oxygen to reduced cytochrome P450 is different from the binding observed with other ligands. Whilst most ligands produce a red-shifted spectrum, the binding of oxygen produces a spectrum with a Soret band maximum at 419 nm (Ishimura et al 1971). This Soret band at 419 nm is close to that observed with myoglobin (418 nm), but very different from chloroperoxidase, which contains a Cys group in the anionic form (RS⁻) as the proximal ligand, where the band is at 430 nm. Based on these observations, it seems reasonable to assign oxy P450 to conformer II (see also section 4.1). This is further supported by the evidence that in microsomal cytochrome P450 oxygen reacts preferentially with conformer II of the ethyl isocyanide complex (Imai and Sato 1966b; 1968b-c) and that lowering the pH promotes the reaction of oxygen in competition with carbon monoxide (Ichikawa et al 1967). It has also been suggested that this conformational change is involved in a transfer of the second electron (Tsubaki et al 1989).

1.10 Thromboxane synthase and allene oxide synthase.

Both thromboxane and allene oxide synthase enzymes lack the conserved amino acids Thr/Ser close to the active site and are involved in reactions that do not involve molecular oxygen. It therefore suggests that the Thr residue plays an important role in the binding of oxygen in the cytochrome P450 enzymes.

Thromboxane A₂ is a potent substance synthesised in the body by blood platelets and is responsible for the constriction of blood vessels as well as platelet aggregation. Thromboxane A₂ is derived from prostaglandin H₂ by the action of the enzyme thromboxane synthase as indicated by the following reaction:-
Thromboxane synthase is classed as a cytochrome P450 type enzyme due to its ability to display a broad spectrum with a band at 450 nm when it is reduced with sodium dithionite in the presence of carbon monoxide (Ullrich et al 1981). The binding of carbon monoxide to reduced purified enzyme produced a single peak at 450 nm, whilst the microsomal preparation produced a spectrum consisting of two peaks at 427 and 450 nm (Haurand and Ullrich 1985), with the formation of these peaks occurring very slowly (30 minutes). As well as carbon monoxide, many nitrogen containing compounds such as pyridine and imidazole have been shown to bind to thromboxane synthase both in the ferric and ferrous forms (Hecker et al 1986).

Allene oxide synthase is located in chloroplasts and is responsible for the conversion of lipoxygenase derived fatty acid hydroperoxides to unstable allene epoxides as indicated by the following reaction:-
1.11 Cytochrome P420.

The pH-dependent conformational changes observed when isocyanides and pyridine bind to reduced cytochrome P450 are distinct from the almost irreversible change from P450 to the so called P420, which probably involves replacing the Cys ligand with His as the proximal ligand (Wells et al 1992). The conversion of microsomal cytochrome P450 to cytochrome P420 has been shown to occur with a variety of reagents, including urea, proteases, sulfhydryl reagents or detergents (Cooper et al 1965; Mason et al 1965). Cytochrome P420 is classed as the denatured form of cytochrome P450 and was first observed by Omura and Sato in 1964. Cytochrome P420 can bind carbon monoxide like cytochrome P450, except that the Soret peak is at 420 nm and not at 450 nm. Cytochrome P420 can also bind ethyl isocyanide in the reduced state, but unlike cytochrome P450 only one Soret peak was observed at 430 nm and appeared to
be pH independent regardless of the preparation (Peisach and Mannering 1985).
Detailed Raman and absorption spectra have suggested that cytochrome P420
haem is in equilibrium between a high spin five coordinate state and a low spin
state both in the ferric and ferrous oxidation states (Wells et al 1992).

1.12 Chloroperoxidase.

Much of our understanding of the peroxidase enzymes has been based on
studies of the pH dependency of rate constants and ligand binding constants
with horseradish peroxidase, chloroperoxidase, mutants of cytochrome c
peroxidase and protein free microperoxidase 8 (MP8).

Chloroperoxidase like cytochrome P450 contains a Cys residue in its anionic
form (RS-); all other known peroxidases possess a proximal histidine. The
spectral properties of chloroperoxidase are more akin to those of the cytochrome
P450 enzymes than to other peroxidases (Ortiz de Montellano 1987). These
spectral properties include the presence of a red shifted Soret band at 430 nm in
the reduced spectrum. Equilibrium binding studies of exogenous ligands and
halides to the active site haem iron of chloroperoxidase have been carried out
over the pH range 2 to 7 (Sono et al 1986). The titration results showed that
for the ferric enzyme and weak acidic ligands (pKa > 3), such as cyanide,
binding to the enzyme occurred in their neutral (protonated) form followed by
deprotonation upon ligation to the haem iron. In contrast, strongly acidic ligands
(pKa < 0) such as thiocyanate bind in their anionic (deprotonated) form with an
additional proton to the acid form of the enzyme.

In addition to being a typical peroxidase, chloroperoxidase can catalyse the
peroxide dependent halogenation of organic substrates utilising halide anions such
as Cl-, Br- and I- as the source of the halogen atom (Thomas et al 1970), and
can act as a catalase by disproportionating hydrogen peroxide to oxygen and water (equation 6). All these reactions involve the formation of an intermediate termed compound I

\[
\text{Catalase} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I} + \text{H}_2\text{O}
\]

\[
\text{Compound I} + \text{H}_2\text{O}_2 \rightarrow \text{Catalase} + \text{O}_2 + \text{H}_2\text{O}
\]  

It has been shown that there is a clear distinction between cytochrome P450 reactivity and those of peroxidases (McCarthy and Coon 1983). Thus, if both of these enzyme classes were to operate through a similar iron-oxo intermediate, then these intermediates are somehow profoundly different from one another, both in their modes of formation and in their intrinsic reactivities.

1.13 Haem oxygenase.

The general hydroxylation reaction (equation 1) is always accompanied by the formation of hydrogen peroxide. The further use of this hydrogen peroxide by cytochrome P450 has prompted comparison of the degradation of cytochrome P450 with the haem oxygenase enzyme.

\[
\text{RH} + \text{H}_2\text{O}_2 (\text{O}_2 + 2\text{H}) \Rightarrow \text{ROH} + \text{H}_2\text{O}
\]

Haem oxygenase is a membrane bound enzyme which is involved in the oxidation of haem to biliverdin, and can exist in two forms (haem oxygenase 1 and 2). The reaction of haem oxygenase with haem requires NADPH, molecular oxygen and a reductase, or hydrogen peroxide on its own. Hydrogen peroxide on its own leads to the production of verdoheme, but cannot produce biliverdin from the verdoheme unless NADPH and molecular oxygen are present. In contrast, the reaction of the haem-haem oxygenase complex with meta-chloroperbenzoic acid or tert— butyl hydroperoxide yields a ferryl (Fe(IV)=O) species and a transient protein radical, but does not result in oxidation of haem to verdoheme or biliverdin (Wilks and Ortiz de Montellano 1993). Evidence that the intermediate involved in the reaction of haem oxygenase with hydrogen
peroxide or molecular oxygen and NADPH is a peroxide (FeOOH) was provided by the spectra produced by the action of hydrogen peroxide on this enzyme. The spectrum showed a decrease in intensity of the Soret band, but without a detectable shift in wavelength, while when hydroperoxides such as meta chloroperbenzoic acid and t-butylhydroperoxide were used, a wavelength shift of 1-3 nm (417-414 nm) was observed, which is comparable to the shift in wavelength observed by the action of iodosobenzene on cytochrome P450 (Blake and Coon 1989). The evidence produced by Wilks and Ortiz de Montellano suggest that the degradation of haem oxygenase occurs via a peroxide type intermediate and not a ferryl intermediate.

1.14 Aims.

One of the most intriguing and characterising reactions of cytochrome P450 is the hydroxylation of substrates, in particular alkanes. Two major questions are still unanswered about this reaction;

1) The nature of the intermediate involved
2) The possible role of the Fe$^{2+}$ conformers in these reactions

Since the general hydroxylation reaction is always accompanied by the generation of hydrogen peroxide ($O_2 + 2H + 2e^- \Rightarrow H_2O_2$), and the hydrogen peroxide produced can react with alkanes ($RH + H_2O_2 \Rightarrow ROH + H_2O$), the overall reaction can in principle be studied in two segments. The aim of the work in this thesis was to endeavour to elucidate the nature of the intermediate and to test the effect on the equilibrium stated by Kahl and co-workers (1976), ([RS$^-$ $\Rightarrow$ Fe(II)$ \leftrightarrow X$]$_I$ + $H^+$ $\Leftrightarrow$ [RSH$^+$ $\Rightarrow$ Fe(II)$ \leftrightarrow X$]$_II$), firstly, of increasing the electron density on the iron by increasing the pK of the base (ie comparing $\sigma$ donor power and $\pi$ acceptor qualities of the bases) and secondly, of ligands which may model a key feature of coordinated oxygen.
In order to investigate the nature of the key intermediate, a suitable source of cytochrome P450 was required. The enzyme chosen for use was the P450 isoform derived from rabbit liver induced with phenobarbital (CYP2B4). Rabbit CYP2B4 enzyme was used due to its ease of extraction and purification and the fact that apart from cytochrome P450cam, it is the most studied. The substrate chosen for studying the hydroxylation reaction was lauric acid which is hydroxylated in the ω, ω-1 and ω-2 positions and can be considered as a solubilised alkane. The change in ratio between these metabolites may provide an additional useful probe of changes around the active site, including the nature of the key intermediate.

The reaction of lauric acid with iodosobenzene and hydrogen peroxide is known to be accompanied by the degradation of the haem (Blake and Coon 1989; Vaz et al 1991). The intermediate involved in this reaction is thought to vary depending on the oxygen donor used. In order to test this theory, iodosobenzene was used to produce the intermediate which exhibited a spectral absorbance maximum at ~390 nm, which was proposed to be the ferryl intermediate (Blake and Coon 1989). This ferryl intermediate will then be used in the hydroxylation reaction, and the products generated would then be compared to those produced by hydrogen peroxide. The action of hydrogen peroxide on cytochrome P450 has remarkable similarities to the action of hydrogen peroxide on haem oxygenase, where the enzyme is degraded without a shift in wavelength and the formation of a spectral intermediate (Wilks and Ortiz de Montellano 1993). It has been suggested that the degradation of haem oxygenase occurs via a peroxide type intermediate and not via a ferryl intermediate. pH-dependent studies involving hydrogen peroxide might provide some additional evidence concerning the nature of the key intermediate, they might indicate whether the intermediate is in its neutral or anionic form (H$_2$O$_2^\cdot$/HO$_2^\cdot$).
Cytochrome P450 is also known to catalyse peroxide type reactions, although much of our understanding of this mechanism of action is based on studies involving peroxidases such as chloroperoxidase, and the study of the pH-dependent rate constants and ligand binding constants. Such studies involving cytochrome P450 are more difficult to carry out and interpret due to a limited range of stability (i.e. between pH 6.5-8.5), and the ambiguity concerning the nature of the sixth ligand in the resting state of the enzyme. Although it is now believed that the sixth ligand is a solvent molecule (H$_2$O or OH$^-$), it is therefore essential to remove any ambiguity about the nature of this ligand as far as possible. The work in this thesis will therefore attempt to identify the ligand using pH dependent studies on the binding constant (K) for one or more neutral bases and an anion to the ferric (Fe$^{3+}$) ion.

The binding to reduced cytochrome P450 can either produce spectra containing one peak (cyanide or carbon monoxide) or spectra containing two peaks (isocyanides or pyridines), which were later denoted conformers I and II. These conformers were found to be in a pH dependent equilibrium with conformer I favoured by high pH and conformer II by low pH (Imai and Sato 1966a-b). It has been proposed by Kahl and co-workers (1976) that the proximal ligand in conformer II is the undissociated thiol (RSH) while conformer I contains thiolate as its proximal ligand. It was initially suggested that single peaked spectrum were produced by hydrophilic compounds (CN or CO) with the peak corresponding to conformer I or II, while others have proposed that the formation of the two banded spectrum is caused by the more hydrophobic compounds which occur in a pH equilibrium (Imai and Sato 1967a). More recently it was suggested that compounds such as phosphonites bearing a strong π acceptor ability in conjunction with a lipophilic nature produced the double spectra (Dahl and Hodgson 1978a). The suggestion that π bonding is responsible for the production of the double spectra cannot be validated, since it
is known that amines which cannot \(\pi\) bond can produce double spectra on binding to reduced cytochrome P450 (Jefcoate 1969b). The production of the double Soret spectra could therefore be due to the nature of the environment in the active site and not \(\pi\) bonding.

Recent work carried out on the coordination of three families of nitrogen bases (amines, azines and azoles) by both an Fe(III) porphyrin (Hamza and Pratt 1994a) and a Co(III) corrinoid (Hamza and Pratt 1994b) have shown that the equilibrium constant (K) for coordination increases with the basicity pK of the ligand according to the following relationship: \(\log K = a\cdot pK + b\), (with similar values of \(a\) for all three families but values of \(b\) falling in the following order: azoles>azines>amines). The values of \(\log K\) may be further increased in the case of hydrazine, hydroxylamine and pyridazine through operation of the so called \(\alpha\)-effect. This is the term originally applied to the enhanced nucleophilic reactivity (in catalysed ester hydrolysis) observed when the donor atom is attached directly to another electronegative atom carrying one or more lone pair of electrons (Edwards and Pearson 1962). Furthermore, recent work has also shown that the binding of thiols and thiolates to Fe(III) produces a value of \(a=0\) (i.e binding constants are essentially independent of the pK) which indicates that both RSH/RSe® are readily accessible as ligands in the physiological range in support of Kahl's suggestion (Norris and Pratt in preparation).

The postulated equilibrium between the thiolate/thiol proximal ligands \([RS^- \Rightarrow Fe(III) \Leftrightarrow X]_I + H^+ \Leftrightarrow [RSH^+ \Rightarrow Fe(II) \Leftrightarrow X]_II\) will be investigated using a series of nitrogen bases, firstly by increasing the electron density on the Fe (by increasing the pK of the base) and, secondly, using ligands which may model a key feature of coordinated oxygen. The binding of oxygen is in fact known to be anomalous producing a single peak at 419 nm in the reduced spectrum, which is close to that of myoglobin (418 nm). It is expected that coordinated
oxygen will show the α-effect (Taube 1986) and that strong hydrogen bonding may be an associated effect. It is thought that hydrazine, hydroxylamine and pyridazine may therefore provide "models" for this aspect of coordinated oxygen and for investigating the α-effect.

It has been suggested that the conserved distal Thr/Ser residues may be involved in the binding of oxygen (Song et al 1993) and the addition of the second electron in the catalytic cycle (Gerber and Sligar 1994). In order to investigate this, a P450 type enzyme that lacks these two residues will be extracted from blood platelets and the binding of certain ligands to this enzyme will be investigated. The enzyme used for this will be thromboxane synthase which handles endogenous hydroperoxides in reactions which require no oxygen.
CHAPTER 2

Purification and characterisation of CYP2B4
2.1 INTRODUCTION.

Hepatic microsomal cytochrome P450 plays an important role in the oxidation of endogenous substrates such as steroids, in the detoxification of xenobiotics, and in the activation of many different drugs and environmental agents to toxic, mutagenic and carcinogenic forms (Gillette et al 1972). In untreated animals cytochrome P450 is the most abundant membrane protein of liver microsomes. Levels of hepatic microsomal cytochrome P450 may be increased by the administration of many drugs, pesticides, carcinogens, hydrocarbons and anaesthetics (Nebert 1979), so that cytochrome P450 represents greater than 10% of the total microsomal protein (Ryan et al 1979).

In order to further investigate the ligand exchange reactions of cytochrome P450 using optical spectroscopy, and to investigate the action of hydrogen peroxide on cytochrome P450, a purified form of the enzyme had to be obtained. This form of the enzyme had to have a well defined range of catalytic activity. The form of cytochrome P450 that fits the above criteria was CYP2B4, the cytochrome P450 isoform induced by pretreatment with phenobarbital. The microsomal content of CYP2B4 on induction by phenobarbital is known to increase 2-3 fold, with a concomitant increase in the level of other microsomal proteins such as NADPH-cytochrome P450 reductase. This form of cytochrome P450 is readily isolated from rabbit liver microsomes by procedures developed by Coon and co-workers 1978, and can be obtained in a good yield of high purity.

During the purification process outlined in figure 2.1, a few problems were encountered, especially with regards to the yield of pure enzyme and in the purification of the reductase fraction. The final yield of pure enzyme was less than predicted when compared to yields obtained by other authors (Coon et al
1978). The low yield was mostly due to the loss of protein after the hydroxylapatite column step. Due to the poor yield obtained with the hydroxylapatite column, it was decided to omit this hydroxylapatite column step and to use the protein eluate from the previous chromatography step, termed partially purified cytochrome P450 in the experiments outlined in the following chapters alongside the pure CYP2B4 already obtained.

The purification of the NADPH-cytochrome reductase fraction was undertaken using the methodology employed by a number of different laboratories with no major problems (Yasukochi and Masters 1976). However, during the purification process outlined in this chapter, the pure reductase fraction remained tightly bound to the 2' 5' ADP column obtained from Pharmacia, and could not be eluted in-spite of changing the strength or pH of the eluting buffer. A similar problem was experienced using the same column by Prof. J. B. Schenkman (personal communication 1995).

The characterisation of the pure and partially purified forms of CYP2B4 was carried out using an assay specific for CYP2B4 involving the dealkylation of pentoxyresorufin in addition to gel electrophoresis. Gel electrophoretic techniques were used, especially in the case of the partially purified preparation to assess the proportion of the CYP2B4 subunit and CYP1A2 subunit in this preparation. The O-depentylation of pentoxyresorufin may be affected by many factors such as the absence or presence of detergent in the reconstituted system and formation of the lipid micelles. The formation of lipid micelles is crucial for the maximum rate of dealkylation to occur, as incomplete micelle formation prevents interaction between cytochrome P450, NADPH-cytochrome P450 reductase and substrate. The presence of high concentrations of detergent has been shown to inhibit the dealkylation of pentoxyresorufin (Rietjens et al 1988), therefore the presence of small amounts of detergent may affect the rate of dealkylation.
Figure 2.1: PURIFICATION OF CYTOCHROME CYP2B4

Microsomes (100%)

Polyethylene glycol precipitation (30%)

6-8% 8-10% 10-12% 12-14%

DEAE cellulose column

(11%) Cytochrome P450 Fraction

Reductase and b5 Fraction

Hydroxylapatite column

2',5' ADP column

70mM Fraction (1.1%)

2'AMP fraction containing pure reductase.

CM Sepharose column

"PURE CYP2B4"
2.2 MATERIALS.

All reagents used were of at least analytical grade and were obtained from the following sources:

**Amicon Instruments Company.** (USA)
PM 30 Diaflo ultrafilters.

**British Drug House Ltd.** (Poole, Dorset, UK)
Ammonium persulphate, copper sulphate, dipotassium hydrogen orthophosphate, disodium hydrogen orthophosphate, EDTA, glycine, potassium dihydrogen orthophosphate, potassium sodium tartrate, sodium carbonate, sodium dihydrogen orthophosphate, sodium dithionite, sodium dodecyl sulphate (SDS), sodium hydrogen carbonate, sodium pyrophosphate.

**British Oxygen Company.** (Guildford, UK)
Carbon monoxide.

**Calbiochem Biochemicals.** (Nottingham, UK)
Aquacide III.

**Fisons Scientific Equipment.** (Loughborough, Leicestershire, UK)
Acetic acid (glacial), glycerol, hydrochloric acid, methanol, potassium chloride, sodium hydroxide.

**Gibco.** (Paisley, UK)
Bluprint fast page stain.
Molecular Probes. (Eugene, Oregon, USA)
Pentoxyresorufin.

National Diagnostics. (Aylesbury, Buckinghamshire, UK)
Acrylamide, bisacrylamide.

Pharmacia. (St. Albans, Hertfordshire, UK)
All column equipment.

Sigma Chemical Company LTD (Poole, Dorset, UK)
Butylated hydroxytoluene, bovine serum albumin, cholic acid (sodium salt), CM Sepharose, DEAE 52 cellulose, Folin-Ciocalteau reagent, hydroxylapatite (granulated), L-α-dilauroyl phosphatidyl choline, NADPH, prestained molecular weight markers, polyethylene glycol (6000-8000), sodium phenobarbital, TEMED, tergitol (NP-10), tris (trizma base).
2.3 METHODS.

2.3.1 Preparation of column chromatography media.

DEAE Cellulose Anion exchange.

The required amount of DEAE cellulose anion exchange medium (1 part medium to 3 parts distilled water w/v) was suspended in distilled water and gently swirled. After allowing the suspension to settle, the 'fines' were decanted and the procedure repeated twice using 10 mM Tris-HCl buffer pH 7.5. The medium was ready for pouring into the column apparatus.

CM-Sepharose.

The above procedure was also used for the preparation of this medium except that 10 mM potassium phosphate buffer pH 7.5, was used as the wash medium instead of the Tris-HCl buffer.

Hydroxylapatite.

Hydroxylapatite (Type III) medium (1 part medium to 3 parts buffer w/v) was prepared by suspending the required amount of the powered matrix in 3 volumes of the starting buffer, 10 mM phosphate pH 7.5 (4 parts potassium to one part sodium). The suspension was gently swirled and after allowing to settle, the 'fines' were decanted and the procedure repeated 3-4 times to remove most of these 'fines'. The medium was then poured into the column apparatus.
2.3.2 Column preparation.

All of the column apparatus used during the purification of CYP2B4 were prepared in the same way. All the seals were greased with silicon grease to prevent leaks and to enable a continuous and steady flow of the eluting buffer.

After the construction of the column apparatus, an aliquot of buffer to be used was poured into the bottom of the empty column. The stirred medium was then poured into the column at a steady rate to avoid the production of convection currents and allowed to settle overnight. A column top was attached and connected via a piece of tubing to a reservoir containing the buffer to be used. The buffer was now allowed to run through the column at a fixed flow rate until a constant column bed height was obtained. The column was washed with buffer until the pH of the eluent was identical to the starting pH of the buffer, the column was now ready for use.
2.3.3 Purification of hepatic microsomal CYP2B4 from phenobarbital treated rabbits.

Animals and animal pretreatment.

Eight male New Zealand white rabbits (Av. wt 2.2 Kg) were obtained from the animal breeding unit (University of Surrey). The animals were maintained on drinking water containing 0.1 % (w/v) sodium phenobarbital solution (adjusted to pH 7.0 with HCl prior to use) for 5 days. After 5 days the animals were fasted overnight prior to killing, in order to minimise the amount of glycogen present in the liver.

Preparation of hepatic microsomes.

The methodology employed for the preparation of CYP2B4 was based on a procedure developed by Coon et al (1978), and was carried out at a temperature of 4 °C. All the buffer solutions contained 20% (v/v) glycerol, 1.0 mM EDTA and were at pH 7.5, unless otherwise stated.

The animals were killed by cervical dislocation. The livers were removed immediately, the gall bladders excised and the livers blotted dry. The livers were subsequently pooled and weighed (688g). The combined liver preparation was then scissored minced and homogenised in 4 volumes of 0.1 M Tris-HCl buffer pH 7.5, containing 0.1 M KCl, (no glycerol) using a Waring blender at the highest speed (2 x 30 seconds). The homogenate was then centrifuged at 12,000g (9000 rpm) for 30 minutes using a 6 x 250 ml aluminium angle head rotor in a Beckman J2-21 centrifuge. The supernatant was decanted and centrifuged at 100,000 g (38,000 rpm) for one hour in a Beckman LS-65 ultracentrifuge using a Ti 60 rotor. The resulting pellets were pooled and
homogenised in 0.1 M sodium pyrophosphate buffer (no glycerol) using a motor driven Potter-Elvehjem glass teflon homogeniser. The pyrophosphate wash was carried out to remove any remaining haemoglobin present in the sample. The pyrophosphate homogenate was centrifuged at 100,000 g (38,000 rpm) for one hour using the same rotor as before. The resulting dark red pellets were hand-homogenised in 50 mM Tris-HCl buffer containing glycerol to a final volume of 500 ml. The homogenate was stored in 5 x 100 ml aliquots overnight at -70 °C.

Solubilisation of microsomal proteins.

The thawed homogenate (100 ml) was mixed with 250 ml of 0.2 M Tris-HCl buffer pH 7.5, containing EDTA (2.0 mM) and butylated hydroxytoluene (0.04 mM) and made up to 500 ml after cholate addition. A 10 % (w/v) sodium cholate solution was added dropwise from a burette with stirring to the microsomes in an ice bath under nitrogen, to give a final cholate to protein ratio of 3:1 and the mixture then stirred for a further 30 minutes. The solution was centrifuged at 100,000 g (38,000 rpm) for 45 minutes in an ultracentrifuge to remove any unsolubilised microsomal protein. The resulting supernatant was decanted and 50 % (w/v) polyethylene glycol (PEG) was added slowly from a burette to the supernatant on ice with continuous stirring, to obtain a 6 % final concentration. Following PEG addition the solution was left to stir for 10 minutes and then centrifuged at 12,000 g (9,000 rpm) for 20 minutes in a Beckman J2-21 centrifuge. Further PEG was then added to the supernatant, and the fractions precipitated from 6-8 %, 8-10 %, 10-12 % PEG were collected by centrifugation. The resulting pellets were resuspended in 50 ml of 10 mM Tris-HCl buffer containing glycerol and EDTA, using a hand held homogeniser. The five 8-10 % PEG fractions which contained the most cytochrome P450 (see section 2.3.10) were combined and adjusted to 0.5 % (v/v) tergitol. This combined fraction was dialysed against 10 mM Tris-HCl buffer containing
glycerol, EDTA and 0.5% (v/v) tergitol.

2.3.4 Diethylaminoethyl (DEAE) cellulose anion exchange chromatography.

The dialysed fraction resulting from PEG precipitation was concentrated, using an Amicon ultrafiltration cell with PM 30 Diaflo ultrafilters, down to a working volume (100 ml) and was then loaded onto a DEAE cellulose column (2.6 x 20 cm) previously equilibrated with 10 mM Tris-HCl buffer pH 7.5. The bound cytochrome P450's proteins were eluted using 10 mM Tris-HCl buffer, containing 20% (v/v) glycerol, 0.5% (v/v) tergitol. The eluate was continually monitored at A

2.3.5 Hydroxylapatite column chromatography.

The pooled CYP2B4 from the DEAE cellulose column was dialysed against 10 mM phosphate buffer pH 7.5, containing 20% (v/v) glycerol, 0.3% (v/v) tergitol and 1 mM EDTA. This dialysate was then applied to two hydroxylapatite columns (2.6 x 10 cm) in parallel which had been previously equilibrated with the dialysis buffer, but containing only 0.1 mM EDTA. Once loaded, the column was washed with 50 mM phosphate buffer, and elution of the protein was achieved by increasing the buffer strength to 70 mM with a flow rate of 3 ml/hr which was achieved using a peristaltic pump. The eluate was monitored and all the fractions having an absorbance greater than 0.12 absorbance units at A

37
content could be measured. SDS-polyacrylamide gel electrophoresis was then carried out to establish the purity of the fraction.

2.3.6 CM-sepharose column chromatography.

This method of chromatography was used to desoap (removal of tergitol) the protein fraction. The concentrated fraction from the hydroxylapatite column was dialysed against 20% (v/v) glycerol and then applied to a CM-sepharose column (1 x 5 cm), which had been previously equilibrated with 10 mM potassium phosphate buffer containing 20% (v/v) glycerol. The column was washed with the equilibrium buffer until the absorbance of the eluent at A$_{280}$ nm reached zero absorbance units, indicating that all the tergitol had been removed from the sample. The protein was eluted from the column using 300 mM potassium phosphate buffer containing glycerol 20% (v/v). The eluted CYP2B4 was pooled by eye as it was eluted from the column in four distinct phases:- a) A very concentrated red phase (5 ml), b) A yellow/orange phase (25 ml), c) A pale yellow phase (30 ml) and d) colourless buffer. This indicated that most of the CYP2B4 was present in fraction a, whilst fractions b and c contained minor amounts. Fractions a, b and c were combined and concentrated down (see section 2.3.9) under nitrogen divided into 1 ml aliquots and then stored at -70 °C until required.

At each stage of the purification process, 1 ml samples were taken for the determination of total cytochrome P450 content and protein content. SDS-polyacrylamide gel electrophoresis was carried out on the final fraction to assess purity of the purified fraction. In addition, densitometry was used to quantify the purity of the purified fraction using a Shimadzu CS-900 Dual-wavelength flying spot scanner linked to a Qume 835 personal computer and Citizen Swift 24 printer.
2.3.7 Purification of NADPH cytochrome P450 reductase.

NADPH cytochrome P450 reductase was routinely purified as a by-product from the purification of phenobarbital-induced CYP2B4. The methodology devised was similar in principle to that described by Yasukochi and Masters (1976). All procedures were carried out at 4 °C.

NADPH cytochrome P450 reductase and cytochrome b5 were eluted from the DEAE cellulose column after the CYP2B4 had been removed, by washing the column with 10 mM Tris-HCl buffer pH 7.5, containing 20% (v/v) glycerol, 0.5% (v/v) tergitol, 1.0 mM EDTA, 0.05 mM dithiothreitol (DTT) and 350 mM KCl. The eluate was continually monitored at $A_{417}$ nm, and those fractions that contained 30% of the highest reductase value were pooled and concentrated to 20 mls. The pooled fractions were then dialysed against two successive 3 litre volumes of 10 mM potassium phosphate buffer pH 7.7, containing 20% (v/v) glycerol, 0.1% tergitol (v/v), 0.01 mM EDTA, 0.2 mM DTT over a 24 hour period.

2.3.8 N6-(6-Aminohexyl)-adenosine 2',5'-diphosphate sepharose 4B affinity chromatography (2',5'-ADP-Agarose)

A portion of the dialysate (1 ml) was loaded onto a 2',5'-ADP-Agarose column (1 x 8 cm) previously equilibrated with the dialysis buffer. On loading a very faint yellow band appeared at the top of the column indicating the binding of the reductase, whilst cytochrome b5 was eluted. On completion of loading, the column was washed with 200 mM potassium phosphate buffer pH 7.7 containing 20% (v/v) glycerol, 0.02 mM EDTA, 0.2 mM DTT, until the absorbance at $A_{280}$ nm reached zero (tergitol removed). The reductase was then eluted with the 10 mM potassium phosphate buffer containing 20% (v/v)
glycerol, 0.02 mM EDTA, 0.2 mM DTT and 0.7 mM 2' AMP. The concentration of 2' AMP was increased to 4.0 mM to ensure all the reductase had been eluted. The eluate was monitored at 451 nm and the fractions containing the most reductase were pooled and concentrated to approximately 5 ml. The 2' AMP was removed by successive dialysis against 3 litres of 50 mM potassium phosphate buffer, pH 7.7 containing 20% (v/v) glycerol. The reductase was then divided into 1 ml aliquots and stored at -70 °C.

2.3.9 Concentration of the various protein fractions obtained during the purification procedure.

Cytochrome P450 fractions were routinely concentrated during the purification procedure in order to obtain usable stock concentrations, using a) Amicon ultrafiltration for large volumes (500 ml), b) aquacide for small volumes (50 ml). a) A fresh membrane of the required molecular weight exclusion was placed in an ultrafiltration cell (capacity 500 ml) obtained from Amicon instruments Co, USA. The cell was filled with distilled water and under a steady stream of nitrogen at a pressure of 30 psi, the water was forced through the membrane in order to displace the glycerol contained in the membrane pores. This procedure was repeated 2-3 times to ensure complete removal of the glycerol. Once this had been achieved, the protein preparation was introduced to the cell and ultrafiltration was carried out at 30 psi.

b) Visking dialysis tubing (5-24/32 "") obtained from Medicell international limited, London, UK. was boiled three times in distilled water and stored at 4 °C prior to use. The required length of tubing was filled with the protein preparation and placed in a petri dish and then subsequently liberally sprinkled with aquacide (modified polymer of high molecular weight). The petri dish containing the tubing was covered in foil and stored at 4 °C until concentration
of the protein fraction had been achieved.

2.3.10 Spectrophotometric enzyme assays.

All spectrophotometric determinations were carried out using a Kontron-Uvikon model 860 split beam spectrophotometer.

Determination of total cytochrome P450.

Total cytochrome P450 content was determined using the method of Omura and Sato (1964a) based on the Fe$^{2+}$ carbon monoxide adduct. Purified protein fractions were diluted (1:6) with 0.1 M potassium phosphate buffer, pH 7.4. Reduction of the ferric form to the ferrous form was achieved by adding a few granules of sodium dithionite. The protein suspension was divided equally between two 1.5 ml cuvettes and a baseline scan was recorded between 500 - 400 nm. Carbon monoxide was bubbled into the sample cuvette at approximately one bubble per second for 30 seconds and the spectrum between 500 - 400 nm was recorded. Total cytochrome P450 concentration was determined employing the extinction coefficient of 91 mM$^{-1}$cm$^{-1}$ for the absorbance difference $A_{450-490}$nm.

Determination of the total protein content.

Total protein content was determined by the Lowry et al (1951) method using bovine serum albumin (fraction V) as the standard.

Reagents:

- 2 % (w/v) sodium carbonate
- 2 % (w/v) sodium potassium tartrate
- 1 % (w/v) copper sulphate
- Folin-Ciocalteau phenol reagent
Bovine serum albumin solution (500 µg/ml) was diluted with sodium hydroxide (0.5 M) to give a range of standards (0-250 µg) in a total volume of 1.0 ml sodium hydroxide. The purified fractions were diluted (2-20 fold) with sodium hydroxide to give an appropriate concentration in a final volume of 1.0 ml. Copper sulphate, sodium potassium tartrate and sodium carbonate were mixed in the ratio (1:1:100 by volume) immediately before use. A portion (5 ml) of this was added to each tube and allowed to stand for 10 minutes at room temperature. An aliquot (0.5 ml) of Folin-Ciocalteau phenol reagent (50 % v/v) was then added to each tube, which was then immediately vortexed and incubated at room temperature for 30 minutes. After 30 minutes the absorbance was read at 720 nm. The protein concentration was calculated from the standard curve.

Most of the purified preparations assayed by this procedure contained glycerol and tergitol, which are known to cause turbidity. On such occasions, the tubes were centrifuged at 1,800 g (3000 rpm) for 10 minutes to remove the turbidity caused by these compounds. After centrifugation the samples were read as previously described.
2.3.11 Characterisation of the purified CYP2B4 fraction.

Alkoxyresorufin O-dealkylase assay.

The O-depentylation of pentoxyresorufin was carried out using the method of Burke and Mayer (1974). The assay system comprised the following reagents:-

<table>
<thead>
<tr>
<th>Stock concentration</th>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 µg/ml</td>
<td>L-α-dilauroyl phosphatidyl choline.</td>
<td>30 µg/ml</td>
</tr>
<tr>
<td>6.9 nmol/ml</td>
<td>Purified CYP2B4.</td>
<td>0.09 nmol/ml</td>
</tr>
<tr>
<td>40 units/ml</td>
<td>NADPH-cytochrome P450 reductase.</td>
<td>0.5 units/ml</td>
</tr>
<tr>
<td>150 mM</td>
<td>Magnesium chloride.</td>
<td>15 mM</td>
</tr>
<tr>
<td>400 mM</td>
<td>Potassium phosphate buffer, pH 7.8.</td>
<td>50 mM</td>
</tr>
<tr>
<td>1 mM</td>
<td>Pentoxyresorufin.</td>
<td>5 µM</td>
</tr>
<tr>
<td>50 mM</td>
<td>NADPH in 1 % (w/v) NaHCO3.</td>
<td>0.25 mM</td>
</tr>
</tbody>
</table>

Components were added in the order given above at 4°C and made up to a final volume of 2 ml with distilled water. All components in the above incubation system were pre-incubated at 37°C for 5 minutes. After 5 minutes the reaction was initiated by the addition of NADPH. The fluorimetric assay was performed on a Perkin Elmer LS-5 luminescence spectrophotometer set at an excitation wavelength of 510 nm and emission wavelength of 586 nm with excitation and emission slit widths of 10 nm and 2.5 nm respectively. A baseline of fluorescence was recorded prior to the initiation of the reaction by the addition of NADPH. The reaction was continually monitored until a measurable gradient
was obtained. Initial rate was ascertained from the slope. Calibration was carried out using aliquots (10 µl) of 0.1 mM resorufin.

Discontinuous SDS-polyacrylamide slab gel electrophoresis.

Discontinuous SDS-polyacrylamide slab gel electrophoresis was carried out as described by Laemmli et al. (1970), using the following buffers:

Lower gel buffer: 1.5 M Tris-HCl pH 8.8, containing 0.4% (w/v) sodium dodecyl sulphate (SDS).

Upper gel buffer: 0.5 M Tris-HCl pH 6.8, containing 0.4% (w/v) SDS.

Electrode buffer: 25 mM Tris-HCl pH 8.3, containing 192 mM glycine and 0.1% (w/v) SDS.

Sample buffer: 62.5 mM Tris-HCl pH 6.8, containing 2.3% (w/v) SDS, 15% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 5% (v/v) mercaptoethanol.

Discontinuous SDS-polyacrylamide slab gel electrophoresis was carried out using a Hoefer SE 400 slab gel unit. The glass electrophoresis cassette was cleaned using acetone, assembled and greased with vaseline to aid the formation of a leakproof seal at the bottom edge of the unit. The unit was placed in a vertical casting stand and tightened onto a silicon gasket to achieve a leakproof seal. The lower gel was prepared using acrylamide gel (8.11 ml), bisacrylamide (3.38 ml), lower gel buffer (6.25 ml), 10% (w/v) ammonium persulphate (125 µl) and distilled water (7.14 ml). Immediately prior to pouring the gel into the cassette, 20 µl of the polymerising agent TEMED was mixed into the gel solution. The
gel was then pipetted into the cassette to a depth of about 10 cm and left to set. A layer of distilled water was placed on top of the gel to ensure an even surface. Once set, the water was removed and an upper gel was prepared using acrylamide gel (1.0 ml), bisacrylamide (0.4 ml), upper gel buffer (2.5 ml), 10 % (w/v) ammonium persulphate (100 µl) and distilled water (6.0 ml). TEMED (20 µl) was added to the gel immediately before it was pipetted into the cassette. A nine well comb was immediately inserted into the upper gel and the gel was allowed to polymerise. When set, the comb was removed carefully revealing nine wells which were then filled with electrode buffer. Samples were diluted to a protein concentration of 0.5 mg / ml with sample buffer and boiled for three minutes in a water bath to ensure denaturation of the protein prior to use. Samples were loaded (20 µl per well) using a 50 µl Hamilton syringe, molecular markers were also loaded. The cassette was then placed into an electrophoresis tank. Electrode buffer was poured into the upper and lower reservoirs of the tank. Electrophoresis was established with a constant current of 20 mA for two hours. Once the dye front was within 3 cm of the bottom of the unit, the cassette was dismantled and the gel removed for staining using the following reagents:

Wash buffer : 45 % (v/v) methanol, 10 % (v/v) acetic acid, 45 % (v/v) distilled water

Staining solution : 8 ml Blueprint fast stain stock solution, 32 ml distilled water, 10 ml wash buffer

Destaining solution : 10 % (v/v) aqueous acetic acid
Prior to staining, the gel was washed in the wash buffer for 10 minutes. This was repeated twice. After the final wash, the gel was submerged in the staining solution and agitated for 20 minutes. After 20 minutes the staining solution was then removed, and the gel was destained using the 10% solution of acetic acid. The destaining process was continued until all traces of stain were removed. The percentage purity of the protein was determined by densitometry on the gel, using a Shimadzu densitometer set to Zig-Zag scan mode.

Preparation of CYP4A and CYP2E microsomal fractions.

Hepatic microsomes of male Wistar albino rats (150-200g, University of Surrey Breeders), which had been pretreated with either clofibrate (250 mg kg\(^{-1}\) i.p. for 3 days), or isoniazid (100 mg kg\(^{-1}\) gavage for 3 days) and killed 24 hours after the last dose were prepared according to a procedure developed by Coon et al (1978).
2.4 RESULTS:

2.4.1 Purification of rabbit CYP2B4

CYP2B4 was purified from rabbits pretreated with phenobarbital as described in section 2.3.1. The purification protocol is represented schematically in figure 2.1 and yields from the purification process are shown in table 2.1.

The total amount of pure CYP2B4 produced during this purification was calculated to be 165 nmol/688g liver, which compares favourably with the yields obtained by other authors which ranged from between 74 nmol to 246 nmol from 300g-868g of liver (Gibson et al 1982, Koop et al 1982). The total amount of pure CYP2B4 produced in this purification process corresponds to a final yield of 0.4% which although low, compares favourably with yields reported by other authors (Van der Hoeven et al 1974, Haugen and Coon 1976, Coon et al 1978, Gibson et al 1982, Koop et al 1982), where the yields ranged from 1.1 to 4.6%. Higher yields may be obtained by either omitting the final desoaping step or by taking all the fractions from PEG precipitation through the purification process. The specific activity of the final preparation was found to be 11.5 nmol CYP2B4/mg protein, whereas the specific activity reported by the authors named above ranged from 11.3 to 20 nmol of cytochrome P450/mg protein. The final yield of CYP2B4 in this purification was lower than that reported by other authors and may be attributed to a substantial loss of the enzyme at the hydroxylapatite column chromatography stage, where a large amount of the partially purified CYP2B4 remained tightly bound to the column. The protein remained bound to the column, even though the ionic strength of the buffer was increased to 500 mM. A possible explanation for the difficulties encountered on elution of the CYP2B4 from this column is that protein-protein interactions may have occurred between the CYP2B4 and CYP1A2 proteins, thus preventing the complete elution and
<table>
<thead>
<tr>
<th>Step</th>
<th>VOL (ml)</th>
<th>PROTEIN CONC (mg/ml)</th>
<th>TOTAL PROTEIN (mg)</th>
<th>CYTOCHROME P450 CONCENTRATION (nmol/ml)</th>
<th>TOTAL P450 (nmol)</th>
<th>SPECIFIC ACTIVITY (nmolP450/mg protein)</th>
<th>YIELD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resuspended homogenate</td>
<td>500</td>
<td>28</td>
<td>14150</td>
<td>89.7</td>
<td>44850</td>
<td>3.2</td>
<td>100</td>
</tr>
<tr>
<td>PEG pttion (8-10% w/v)</td>
<td>308</td>
<td>9.6</td>
<td>3005</td>
<td>44.3</td>
<td>13659</td>
<td>4.6</td>
<td>30</td>
</tr>
<tr>
<td>DEAE column (post dialysis)</td>
<td>520</td>
<td>1.2</td>
<td>613</td>
<td>9.1</td>
<td>5380</td>
<td>7.9</td>
<td>11</td>
</tr>
<tr>
<td>Hydroxylapatite column</td>
<td>66</td>
<td>0.7</td>
<td>44.3</td>
<td>7.5</td>
<td>496</td>
<td>11.2</td>
<td>1.1</td>
</tr>
<tr>
<td>CM Sepharose column</td>
<td>24</td>
<td>0.6</td>
<td>14.4</td>
<td>6.9</td>
<td>165</td>
<td>11.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 2.1. Purification of CYP2B4 from the hepatic microsomal fraction of rabbits pretreated with phenobarbital.
separation of the CYP2B4 from the other cytochrome P450 isozyme (Tamburini et al 1984). Furthermore, it must also be taken into account that the carbon monoxide assay for determination of cytochrome P450 content does not differentiate between different forms of cytochrome P450, so that the value obtained for the steps 1 to 3 (homogenate to hydroxylapatite column) takes into account all cytochrome P450s present and not just the amount of CYP2B4 present. Due to the substantial loss of the CYP2B4 protein on the hydroxylapatite column, all subsequent purification processes were taken to be complete after the DEAE-cellulose step, which gave a final yield of 12 % (10 - 14 %) with a specific activity of 7.9 nmol. This preparation was named the partially purified fraction. Similar experimental results have been obtained with partially purified preparations of CYP2B4 from phenobarbital-induced animals containing up to 95 % of the CYP2B4 protein, with the remaining 5 % CYP1A2, as with preparations containing 6 or 18 nmol/mg of pure protein (Guengerich et al 1975). The 5 % of CYP1A2 present in the partially purified fraction has been shown to have no affect on the binding spectra produced by CYP2B4 in either the reduced or oxidised state (White and Coon 1982).

After the cytochrome P450 proteins had been eluted from the column, purification of the cytochrome b5 and NADPH-cytochrome c reductase which had remained bound to the top of the column was undertaken using the methodology of Yasukochi and Masters (1976). The initial yield of NADPH-cytochrome c reductase from the purification process was found to be very low (0.1 %). This portion of NADPH-cytochrome c reductase was subsequently used for the reconstitution experiment. When the bulk of the concentrated reductase/cytochrome b5 mixture was applied to the same column, after elution of the b5 fraction, elution of the pure reductase fraction was carried out using 2' AMP. However, inspite of using 2'AMP at various concentrations (0.7 - 5 mM), the reductase remained bound to the column. Subsequently, different
methods of elution were attempted; these included NADP at a concentration of 1.0 mM, pH gradient (7.7 - 4.4), salt gradient (0.2 M - 1.0 M), addition of tergitol, and finally 6 M urea. All of these elution methods failed to remove the reductase which seemed to be irreversibly bound to the ADP agarose column. Since the last elution method (6 M urea), failed to remove any of the reductase, a second attempt was made to purify the reductase using the above method, except that purification of the reductase / b5 was attempted using different column packing materials, namely, Sephadex G-75 (Omura and Takesue 1970), DEAE-Sephadex A25 (Dignam and Strobel 1975), hydroxylapatite (Iyanagi et al 1978), and DEAE-22 cellulose (Lu et al 1972). Inspite of using all these different packing materials, the reductase could not be separated from the cytochrome b5 in significant quantity or purity, for subsequent use in reconstitution experiments.

2.4.2 Characterisation of pure and partially purified fractions.

Once all the fractions of partially purified and pure CYP2B4 had been collected and concentrated to an appropriate working volume, characterisation of the enzyme was carried out using the techniques of SDS-gel electrophoresis, optical spectroscopy and fluorimetry.

Examination of the photograph of the gel (figure 2.2) clearly shows the presence of one band in the pure sample and three bands (one major and two minor) in the partially purified sample. The single band in the pure sample and the major band in the partially purified both correspond to the CYP2B4 subunit and appears to have a molecular weight of 49,000, which was calculated using the following molecular weight standards :-
Fig 2.2 A SDS-page gel showing the different stages in the purification of CYP2B4.

Track 1 - Molecular weight markers.
2 - Homogenate.
3 - Partially purified CYP2B4.
4 - Pure CYP2B4.
### Protein Approximate Mr (daltons)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Approximate Mr (daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macroglobulin</td>
<td>180,000</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>116,000</td>
</tr>
<tr>
<td>Fructose-6-phosphate kinase</td>
<td>84,000</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>58,000</td>
</tr>
<tr>
<td>Fumarase</td>
<td>48,000</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>36,500</td>
</tr>
<tr>
<td>Triose phosphate isomerase</td>
<td>26,600</td>
</tr>
</tbody>
</table>

The minor band in the partially purified sample corresponds to CYP1A2 which has an estimated molecular weight of 54,000. Literature values for these two forms of cytochrome P450 range from 48,500 to 50,000 for the CYP2B4 subunit and from 53,000 to 55,300 for the CYP1A2 subunit (Haugen et al 1975, Guengerich 1979, Lu and West 1980, Guengerich et al 1982). A spectral scan of the reduced purified fraction produced a peak at 451 nm and no peak at 420 nm. This indicated that the enzyme was active and contain no denatured form of the enzyme. The oxidised spectrum produced a peak at 417 nm indicating the enzyme was in a low spin state. These observations are in agreement with the values quoted in the literature (White and Coon 1982).

The dealkylation of pentoxyresorufin is the specific assay used for the identification of the major phenobarbital-induced form of cytochrome P450, namely CYP2B4. When the purified CYP2B4 fraction was reconstituted into an assay system containing NADPH-cytochrome c reductase and lipid, dealkylation of the substrate occurred (table 2.2). However, when NADPH-cytochrome c reductase fraction was omitted from the enzyme system, no dealkylation occurred. The amount of dealkylation that occurred in the reconstituted system (423 pmol resorufin/ml (705 pmol/mg protein)) was comparable to the figure reported by other authors (181-1241 pmol resorufin/mg protein) (Lubet et al...
The dealkylation of pentoxyresorufin was used to identify the form of cytochrome P450 protein that had been purified rather than to use it to confirm CYP2B4 induction.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cytochrome P450 nmol / ml</th>
<th>Rate of Dealkylation.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>89.7</td>
<td>1494</td>
<td>16.6</td>
</tr>
<tr>
<td>Complete system (CYP2B4 and reductase)</td>
<td>6.9</td>
<td>423</td>
<td>61</td>
</tr>
<tr>
<td>System with CYP2B4 but without reductase</td>
<td>6.9</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.2 Rates of O-dealkylation of pentoxyresorufin by purified CYP2B4 in a reconstituted system.
2.5 DISCUSSION.

The purification procedure described in this chapter provided an ideal method for the isolation of the major form of phenobarbital-induced rabbit hepatic microsomal cytochrome P450, namely, CYP2B4. Furthermore, the protein was purified to an approximate purity of 93% using this procedure.

The estimated molecular weight of the purified protein as determined by SDS gel electrophoresis using prestained molecular weight markers as indicators of the molecular weight, revealed that the subunit molecular weight of the purified cytochrome P450 protein to be 49,000 which corresponds to the molecular weight of the CYP2B4 protein (Haugen et al. 1975; Lu and West 1980). Furthermore, evidence for the purification of the CYP2B4 isozyme was provided by analysis of the oxidised and reduced spectra of the purified protein which displayed peaks at 417 nm and 451 nm, respectively, indicating that the purified protein was CYP2B4 (White and Coon 1982). Conclusive confirmation of the purification of the CYP2B4 isozyme was obtained by measurement of the rate of O-dealkylation of the substrate; pentoxyresorufin, an activity known to be catalysed by CYP2B4 (Lubet et al. 1984), when the purified protein was incorporated into a reconstituted system in the presence of lipid and purified NADPH cytochrome reductase (table 2.2). Dealkylation of the substrate did not occur in the absence of NADPH-cytochrome reductase. SDS-gel electrophoresis and spectral analysis also revealed that the partially purified fraction contained essentially the CYP2B4 protein.

The enzyme system in liver microsomes which is responsible for the hydroxylation of various substrates has been solubilised and resolved into three components; cytochrome P450, NADPH-cytochrome c reductase and a heat-stable substance called factor B (Lu and Coon 1969). The heat-stable factor has been
identified to be phosphatidylcholine and has been shown to be essential for the reductase-catalysed transfer of electrons from NADPH to cytochrome P450 (Strobel et al 1970). Variations in the rates of reaction using purified cytochrome P450 and different substrates in reconstitution systems have been known to occur (Koop et al 1982). These variations in the rates of reaction between different authors are most likely attributed to the preparation of the reconstitution system. In order for maximal rates of catalysis to occur the three components in a reconstitution system must form vesicles which resemble the initial microsomal environment with regard to bilayer structure, phospholipid composition, surface charge, protein:l lipid ratio, and cytochrome P450:NADPH-cytochrome P450 reductase ratio (Bosterling et al 1979). If this is not achieved a variation in the rates of activity can occur. The lipid, phosphatidylcholine is used in the majority of reconstituted systems and has been shown to be effective in stimulating substrate conversion in these systems (Strobel et al 1969). Evidence has also shown that phospholipids, in particular phosphatidylcholine plays an important role in the binding of substrate to the active site of the cytochrome P450 molecule thus indicating that the substrate binding site of cytochrome P450 is closely associated with membrane phospholipids (Taniguchi et al 1984).

The purification of the reductase fraction was undertaken following the methodology of Yasukochi and Masters (1976). However, during the purification process the isolated reductase fraction remained bound to the 2'5' ADP agarose column. This irreversible binding to the 2'5' ADP agarose column has also been reported by other authors (French and Coon 1979, Schenkman personal communication 1995). In the case of French and Coon, 15% of the reductase fraction loaded on to the column was found to remain bound after elution with 2' AMP, but was subsequently eluted using NADP+. The binding of this reductase fraction to the column after 2' AMP treatment was attributed to
differences in the lipid environment in the column matrix (French and Coon 1979). However, although in this case the reductase was eluted, in the purification process outlined in this chapter and the process attempted by Schenkman, the reductase fraction was found to be irreversibly bound to the column, inspite of using different elution methods. The irreversible binding was most probably due to the formation of an irreversible bond between the column matrix and the reductase protein.
CHAPTER 3

Reactions of oxidised CYP2B4 involving hydrogen peroxide.
3.1 INTRODUCTION.

Most cytochrome P450 reactions proceed with the stoichiometry characteristic of monooxygenases:

\[ \text{NAD(P)H} + \text{O}_2 + \text{RH} \rightarrow \text{NAD(P)} + \text{H}_2\text{O} + \text{ROH} \quad (1) \]

where RH is the substrate and ROH the product (Guengerich 1991). The substrates available for this hydroxylation reaction include alkanes and long chain fatty acids. Cytochrome P450 can also undertake peroxidase type reactions when they react with alkyl hydroperoxides and hydrogen peroxide. The mechanism of action is represented by the following equation, where R is an alkyl group or a hydrogen atom.

\[ \text{RH} + \text{ROOH} \rightarrow \text{R'OH} + \text{ROH} \quad (2) \]

The nature of the key intermediate involved in the NADPH and molecular oxygen mediated catalytic cycle (fig 1.1), has been debated for a number of years. There are two main theories concerning this intermediate; the intermediate has been proposed to be either a single oxygen unit or a two oxygen unit. The single oxygen unit theory involves the formation of a ferryl intermediate (FeO)\(^{3+}\), which could also be derived from a single oxygen donor such as iodosobenzene. This ferryl derivative can be detected spectrally under certain conditions and is similar to compound I formed during peroxidase reactions (Blake and Coon 1989). The two oxygen unit intermediate theory was initially developed by White and Coon in 1980 and investigated further by studying reactions involving \(17\alpha\)-hydroxyandrogen and cytochrome P450 \(17\alpha\) (Akhtar et al 1994). In both these cases the reactions involved the formation of a two oxygen unit which possess the structure of an iron-bound peroxide (Fe\(^{III}\)-OOH).

The single oxygen unit theory is reasonable since a metal double bonded to an oxygen atom such as Ru=O\(^{3+}\) is known to hydroxylate alkanes (Che et al
The two oxygen unit theory is also reasonable since simple protonated hydrogen peroxide ($\text{H}_3\text{O}_2^+$) is known to hydroxylate alkanes in the presence of superacids such as $\text{FSO}_3\text{H-SbF}_3$, $\text{FSO}_3\text{H}$, $\text{H}_2\text{SO}_4$ and HF (Olah et al 1977). With this in mind hydrogen peroxide attached to a Lewis acid (eg. $\text{Fe}^{3+}$) could also probably hydroxylate alkanes. The possibility exists that the key intermediate could be either and that both may be used in different circumstances. Therefore the need exists for a direct test of the single oxygen unit against a two oxygen unit in one or more reactions.

The characteristic monooxygenase reaction (1) is always accompanied by the production of hydrogen peroxide and degradation of the porphyrin ring.

\[ \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \Rightarrow \text{H}_2\text{O}_2 \quad (3) \]

\[ \text{H}_2\text{O}_2 + \text{RH} \Rightarrow \text{H}_2\text{O} + \text{ROH} \quad (4) \]

Cytochrome P450 can also catalyse the reaction of hydrogen peroxide directly with alkanes (4). This reaction is also accompanied by self-degradation by the hydrogen peroxide of the porphyrin ring (Karinuza and Mengazetdinov 1991). Due to the destructive nature of hydrogen peroxide very little work has been carried out on the degradation of the porphyrin ring. This is probably because the degradation was thought to be a nuisance, and its presence has detracted from studying reaction (4). The reactions (3) and (4) involved in the formation and further use of the hydrogen peroxide allows the study of reaction (1) as 2 segments and hence emphasises the need to pursue further the investigation of the porphyrin ring degradation by hydrogen peroxide.

The reactions of hydrogen peroxide involving cytochrome P450 allow one to compare the reactions of cytochrome P450 with peroxidases such as chloroperoxidase, which also contains a Cys residue as its proximal ligand, and an oxygenase, namely haem oxygenase, which contains a His residue as its proximal ligand. The comparison of cytochrome P450 with chloroperoxidase led
to the proposal for a ferryl intermediate which is similar in structure to compound I in peroxidases. This in turn led to a search for other oxygen donors other than hydrogen peroxide such as iodosobenzene. It must be remembered that the ferryl intermediate is never observed during the reaction due to its very short half-life, although it can be prepared by the reaction of cytochrome P450 alone with iodosobenzene (Blake and Coon 1989). The reaction of hydrogen peroxide with haem oxygenase has recently been found not to involve the ferryl intermediate but occurs via a two oxygen unit (Wilks and Ortiz de Montellano 1993). The demonstration of some common features between haem oxygenase and cytochrome P450 could provide indirect evidence for the involvement of a two oxygen unit. While preparation of the ferryl intermediate using iodosobenzene provides a more direct means of testing the proposed single oxygen unit, which rather surprisingly has never been reported with either negative or positive results.

The understanding of the peroxidase mechanism of action has been based on studies of the pH-dependent rate constants and ligand binding constants, of chloroperoxidase (Sono et al 1986), cytochrome c peroxidase mutants and protein free MP8. Such pH-dependent studies involving cytochrome P450 are more difficult because of the limited range of stability (pH 6.5-8.5); both high and low pH cause denaturation of cytochrome P450 (Ichikawa et al 1967). One ongoing debate concerns the nature of the sixth ligand present in the resting state of cytochrome P450. The nature of this ligand is now agreed to be either a water molecule or hydroxide ion. Some authors have advocated that the sixth ligand is the hydroxide ion, although no direct evidence has been forwarded (Banci et al 1994). Therefore in order to remove as far as possible any ambiguity about the nature of the sixth ligand, the pH-dependency of the binding constants using one or more bases and an anion should be studied.
The aim of the work carried out in this chapter was to study the reactions of hydrogen peroxide involving both the degradation of the porphyrin ring and hydroxylation of an alkane by looking directly at equation (4). The hydroxylation reaction was investigated using what is effectively a solubilised alkane, namely lauric acid. Lauric acid is known to produce different products ($\omega$, $\omega$-1, $\omega$-2) depending on which oxygen donor is used (Romano et al 1988; Ortiz de Montellano et al 1992). Furthermore, it has been shown that lauric acid possesses a finite solubility, which allows one to quantitatively study the effect of substrate concentrations. In addition the production of metabolites and the change in ratio of $\omega$:$\omega$-1:$\omega$-2 may also provide an additional probe for the changes occurring in the active site, which may include identification of the intermediate involved in the hydroxylation reaction. However, the pKa of lauric acid which is thought to be between 4.5 - 5.0 (cf. hexanoic, heptanoic, octanoic acid all 4.9), may affect the pH-dependency of the rate constant and will have to be taken into account when the data is examined. The use of iodosobenzene as an oxygen donor has been investigated by other authors (Blake and Coon 1989), and iodosobenzene will therefore be used to prepare and test the ferryl derivative as a possible intermediate.

The identity of the sixth ligand in the resting state of the enzyme is agreed to be either a water molecule or hydroxide ion. It seems appropriate to undertake work in this chapter to identify the sixth ligand, because if any pH-dependent change is ignored any equilibrium involving the substitution of coordinated water by an anion or base would be expected to be pH-independent. It is however difficult to envisage such an equilibrium involving the substitution of the coordinated hydroxide ion would be pH-independent for both bases and anions. Therefore the identity of the sixth ligand will be investigated using the anion CN$^-$ and the bases N-acetylimidazole and pyridine.
3.2 MATERIALS

These were as cited in section 2.2. Additional chemicals not previously mentioned are given below:-

**British Drug House (Poole, Dorset, UK)**
Potassium cyanide, pyridine

**Fisons Scientific Equipment. (Loughborough, Leicestershire, UK)**
Diethylether

**Radiochemicals Ltd (Amersham, Buckinghamshire, UK)**
$[1^{14}\text{C}]$ Lauric acid

**Sigma Chemical Company (Poole, Dorset, UK)**
Benzphetamine, hydrogen peroxide, iodosobenzene diacetate, lauric acid, N-acetylimidazole

NADPH-cytochrome c reductase was a kind gift from Professor J. B. Schenkman (University of Connecticut Health Center, Connecticut, USA).
3.3 METHODS.

3.3.1 Preparation of pure and partially purified cytochrome P450

The cytochrome P450 used in the following experiments was prepared as previously described in section 2.3.3. The removal of the detergent (desoaping) from the partially purified samples was achieved as described in section 2.3.6.

3.3.2 The binding of nitrogen containing compounds to oxidised cytochrome P450.

Investigations into the binding of nitrogen containing compounds to oxidised cytochrome P450 was carried out using partially purified cytochrome P450 at a concentration of 1.0 μM, using 0.2 M potassium phosphate buffer pH 7.5. After the baseline had been recorded, the P450 suspension was placed in a sample cuvette, autozeroed and the spectrum recorded. The ligand was then added to the sample cuvette, so that the final concentration was 50 mM unless otherwise stated, then the spectrum was re-recorded.

3.3.3 Determination of the binding constant.

In order to obtain the greatest accuracy possible, the bases that produced the greatest shift in wavelength on binding were chosen.

a) nitrogen compounds

The binding constants were determined for the nitrogen containing compounds with ferric cytochrome P450 using the following procedure; Partially purified cytochrome P450 was diluted to give a final concentration of 1.0 μM. After the
baseline had been recorded using buffer, the partially purified cytochrome P450 suspension was placed in the sample cuvette and the spectrum recorded. Various amounts of the ligand were added to the sample cuvette, whilst equal volumes of buffer were added to the reference cuvette. Titration of ligand to oxidised cytochrome P450 was carried out at the following pHs 6.5, 7.5 and 8.5 for all the ligands.

b) cyanide

The following procedure was used to determine the binding constant for potassium cyanide. Partially purified cytochrome P450 was diluted in 0.2 M potassium phosphate buffer, to give a final concentration (1 μM) in the cuvette. A baseline was recorded with buffer, in both the sample and reference cuvettes. Diluted cytochrome P450 was subsequently placed in the sample cuvette, autozeroed and then the absolute (oxidised) spectrum was recorded. Potassium cyanide (KCN) from a 1 M stock solution was subsequently added to the sample, so that the final concentration in the cuvette was 1 mM and the spectrum recorded. Further amounts of KCN were added to the cuvette so that the final concentration was increased by 2.0 mM after each addition. This procedure was carried out at the following pHs: 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. Similar experiments were carried out for pure CYP2B4, both in the presence and absence of phosphatidylcholine at a concentration of 30 μg/ml.

3.3.4 The effect of hydrogen peroxide on cytochrome P450.

A series of experiments using the oxidised cytochrome P450 spectrum were undertaken to investigate the effect of hydrogen peroxide on cytochrome P450. All the experiments were carried out on a Uvikon 860 dual beam spectrophotometer.
These experiments were carried out to ascertain the effect of temperature on the action of hydrogen peroxide on cytochrome P450. The temperatures used were room temperature (25 °C) and the incubation temperature for the measurement of lauric acid activity (37 °C). Furthermore, the effects of hydrogen peroxide concentration on cytochrome P450 was investigated.

Partially purified cytochrome P450 samples, either in the presence or absence of tergitol, were diluted in Tris-HCl buffer, pH 7.0, so that the final concentration was approximately 1.0 μM. An oxidised spectrum of the sample was recorded and hydrogen peroxide was added to the sample cuvette, and the spectrum re-recorded. Scans were repeated at approximately 4 minute intervals for about 40 minutes. This procedure was repeated for all the concentrations of hydrogen peroxide used and at both temperatures under investigation.

The effect of pH and substrate concentration on the action of hydrogen peroxide was carried out using the above experimental procedure. The experiment was carried out at two additional pHs; 6.5 and 7.5. Both the addition of substrates either before or after the addition of hydrogen peroxide was investigated. The substrates investigated included benzphetamine, guaiacol, pentoxyresorufin and the ligand pyridine, all compounds were used at varying concentrations.

3.3.5 The action of iodosobenzene on cytochrome P450.

The effect of low concentrations of iodosobenzene (0.02 - 0.2 mM) was initially investigated at two temperatures, namely 25 °C and 37 °C. These experiments were carried out using both pure and partially purified samples of cytochrome P450. A sample of cytochrome P450 was diluted in buffer pH 7.0, so that the final concentration in the sample cuvette was 1.0 μM. An oxidised spectrum was then recorded between the wavelengths 350 and 500 nm. Various concentrations
of iodosobenzene were subsequently added to the sample, with an equal volume of buffer added to the reference cuvette. The spectrum was repeatedly scanned for up to a period of 4 hours.

3.3.6 The action of hydrogen peroxide on lauric acid hydroxylation.

The identification of the metabolites produced by the action of hydrogen peroxide on lauric acid was determined using the method of Orton and Parker (1982). An incubation system comprising of the following was set up in triplicate.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium chloride</td>
<td>100 µl 15 mM</td>
</tr>
<tr>
<td>Phosphatidylcholine (freshly sonicated)</td>
<td>100 µl 30µg/ml</td>
</tr>
<tr>
<td>Lauric acid (12C)</td>
<td>50 µl 0.05 mM</td>
</tr>
<tr>
<td>[1,14C] lauric acid</td>
<td>10 µl 0.1 µCi</td>
</tr>
<tr>
<td>Pure CYP2B4</td>
<td>50 µl 1.0 µM</td>
</tr>
<tr>
<td>Tris-HCl buffer, pH 7.5</td>
<td>690 µl 50 mM</td>
</tr>
</tbody>
</table>

1 ml

Tubes were preincubated at 37 °C for 5 minutes in a shaking waterbath. The reaction was then initiated by the addition of 15 mM hydrogen peroxide. The tubes were then further incubated for 10 minutes at 37 °C in the shaking waterbath. Termination of the reaction was achieved by the addition of 3 M HCl (200 µl). Metabolites were subsequently extracted into diethylether, this was achieved by the addition of diethylether (10 ml) to each tube. Tubes were then shaken for 10 minutes on an end over end shaker. In order to clarify the phases, the tubes were centrifuged at 2,000 rpm for 15 minutes in a Beckman J-6B centrifuge. Aliquots of the organic phase (8.0 ml) were removed into clean
glass tubes and evaporated to dryness under a stream of nitrogen. The resulting dried residues were then capped and stored at -20 °C until required. The dried residue was later reconstituted in 150 μl of a reconstitution mixture comprising 45 % (v/v) methanol, 55 % (v/v) water and 0.05 % (v/v) acetic acid. An aliquot (100 μl) of this reconstituted mixture, was subsequently injected into a HPLC column (μ bondapak C18 3.9 x 300 mm), which had been previously equilibrated with a methanol / 0.1% acetic acid mixture (45 / 55 %).

In the absence of standards, metabolite identification was carried out by HPLC analysis of the hydroxylaurate metabolites produced by a combination of the NADPH dependent lauric acid hydroxylation system, using clofibrate-induced microsomes and hydrogen peroxide supported pure cytochrome P450 catalysed lauric acid hydroxylation. An aliquot (100 μl) of the hydroxylaurate metabolites and lauric acid, produced by clofibrate-induced microsomes was added to an equal volume of hydroxylaurate metabolites and lauric acid produced by the action of hydrogen peroxide on pure cytochrome P450, and then 100 μl of this mixture was injected into the HPLC. Since it is known that the microsomal system produces the ω and (ω-1) hydroxylaurate metabolites, and the hydrogen peroxide supported pure cytochrome P450 hydroxylations of lauric acid produces (ω-1) and (ω-2) (Romano et al 1988), the ω and (ω-2) positions were identified in relation to the (ω-1) peak.

3.3.7 The effect of various parameters on the action of hydrogen peroxide supported cytochrome P450 catalysed lauric acid hydroxylation.

The effect of a) incubation time was essentially carried out as outlined in section 3.3.6 using pure CYP2B4 with hydrogen peroxide and b) varying hydrogen peroxide concentration with an incubation time of 10 minutes and c)
varying $^{12}$C lauric acid concentration with an incubation time of 10 minutes and a hydrogen peroxide concentration of 15 mM.

3.3.8 Lauric acid hydroxylation using a reconstituted system.

<table>
<thead>
<tr>
<th>component</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>30μg/ml</td>
</tr>
<tr>
<td>Pure CYP2B4</td>
<td>1.0 μM</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase</td>
<td>1 unit</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>15 mM</td>
</tr>
<tr>
<td>Tris-HCl buffer pH 7.5</td>
<td>50 mM</td>
</tr>
<tr>
<td>$[1-^{14}$C] lauric acid</td>
<td>0.1μCi</td>
</tr>
<tr>
<td>$^{12}$C lauric acid</td>
<td>0.05 mM</td>
</tr>
</tbody>
</table>

Incubations were carried out in duplicate. The components were added in the order stated above at 25 °C to give a final incubation volume of 1 ml. The incubation mixture was preincubated at 37 °C for 5 minutes prior to the initiation of the reaction by the addition of 40 μl of NADPH (40 mM). Tubes were incubated for a further 20 minutes to ensure that complete hydroxylation had taken place. The reaction was terminated by the addition of 0.2 ml of HCl (3 M). Diethylether (10 ml) was added to each tube and shaken on an end over end shaker for 20 minutes to ensure the maximum extraction. Clarification of the phases was achieved by centrifugation at 2,000 rpm in a Beckman J-6B centrifuge. After removal of the organic phase (8.0 ml) to clean tubes, evaporation of the diethylether extracts was carried out under nitrogen in order to obtain a dried residue. The residue was subsequently reconstituted and analysed on a HPLC as previously described in section 3.3.6.
3.3.9 The effect of pH on the hydrogen peroxide supported hydroxylation of lauric acid.

The effect of pH on the hydrogen peroxide supported hydroxylation of lauric acid was carried out using pure, and microsomal (phenobarbital induced) cytochrome P450 together with the assay outlined in section 3.3.6. The results from the initial experiments suggested that the optimal experimental conditions were; a 10 minute incubation period, $^{12}$C lauric acid concentration of 0.05 mM and a final hydrogen peroxide concentration of 15 mM. However, when pH 6.5 and 7.0 were used, the concentration of the stock solution of $^{12}$C lauric acid was decreased to 0.5 mM from 1.0 mM due to the insolubility of the substrate at these low pHs. In these experiments the hydroxymetabolites and lauric acid were extracted into diethylether for 30 minutes instead of 10 minutes in order to ensure maximum extraction of the hydroxylaurate metabolites and parent compound.

3.3.10 The effect of tergitol on hydrogen peroxide supported hydroxylation of lauric acid.

Prior to experimental use, tergitol was removed from the partially purified cytochrome P450 samples using the method described in section 2.3.6. The effect of tergitol on the hydroxylation of lauric acid was investigated using the experimental systems outlined in section 3.3.6, except that the pure cytochrome P450 was replaced with samples of partially purified. The cytochrome P450 systems used were:

a) Desoaped samples of partially purified
b) Partially purified samples containing tergitol
c) Desoaped samples with tergitol added at a final concentration of 0.1% (v/v).
3.3.11 The effect of iodosobenzene on lauric acid hydroxylation.

The hydroxylation of lauric acid by iodosobenzene was carried out using the incubation system outlined in section 3.3.6, except that iodosobenzene at a final concentration of 1.0 mM was used to initiate the reaction.
3.4 RESULTS

3.4.1 The binding of ligands to oxidised CYP2B4.

Oxidised CYP2B4, both in the pure and partially purified forms, produces a spectrum with a Soret band at 417 nm (416 - 418), as determined by spectrophotometry. The addition of ligands to the oxidised cytochrome P450 caused the Soret band to be red-shifted (increase in wavelength). The binding of a selection of nitrogen ligands to CYP2B4 was undertaken to determine which ligands would produce the greatest shift in wavelength, and therefore exhibit the greatest accuracy. The ligand that caused the greatest shift in wavelength was the anion cyanide, followed by the azoles, azines and amines (table 3.1). Different concentrations of ligand were used in order to achieve the maximum shift in wavelength possible. The binding of these ligands to CYP2B4 was examined at various pHs and the binding constants determined.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>Wavelength observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>50</td>
<td>420</td>
</tr>
<tr>
<td>Pyrazidine</td>
<td>10</td>
<td>417</td>
</tr>
<tr>
<td>Imidazole</td>
<td>50</td>
<td>421</td>
</tr>
<tr>
<td>N-acetylimidazole</td>
<td>50</td>
<td>422</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>250</td>
<td>421</td>
</tr>
<tr>
<td>Trifluoroethylamine</td>
<td>27</td>
<td>418</td>
</tr>
<tr>
<td>Cyanide</td>
<td>50</td>
<td>436</td>
</tr>
</tbody>
</table>

Table 3.1: The wavelength shift observed when nitrogen bases and cyanide bind to oxidised partially purified CYP2B4 at pH 7.0.
3.4.2 The determination of binding constants for various ligands to oxidised CYP2B4.

Cyanide.

The binding of cyanide (KCN) to oxidised CYP2B4 produced the greatest shift in wavelength (417 - 435), and implied a greater sensitivity in spectral data. These binding experiments were carried out using both partially purified and pure forms of the enzyme. The spectra obtained on the binding of KCN to partially purified CYP2B4 at various pHs are shown in fig 3.1 (A-E). From these spectra good isosbestic points were found at 430 nm. In contrast, the binding of KCN to the pure form of the enzyme produced broken isosbestic points (fig 3.1 (F)). The quantitative determination of the log K was carried out at various pHs by the rapid titration of KCN (1 mM) to CYP2B4; the binding of KCN to the enzyme was instantaneous with no further shift in wavelength with time. These experiments were carried out at least in duplicate and the log K values were determined from log-log plots for several pHs (table 3.2) which gave slopes of n=1 (eg pH 8.5 fig 3.2). From the results obtained a correlation was found between log K and pH for the partially purified form of the enzyme, whilst only values of log K for pure enzyme at pH 8.0 and 8.5 gave the same slope. There was deviation below pH 8.0 and also above pH 8.5 (fig 3.3).
Fig 3.1 Spectra produced on the binding of potassium cyanide to oxidised partially purified CYP2B4 at various pHs. Curve A, pH 7.0, Curve B, pH 7.5, Curve C, pH 8.0, Curve D, pH 8.5, Curve E, pH 9.0, and on the binding to pure CYP2B4 at pH 8.5 (Curve F).
Fig 3.2 Log-log plot of cyanide concentration against change in absorbance (ΔA) using partially purified samples of CYP2B4 at pH 8.5.

<table>
<thead>
<tr>
<th>pH</th>
<th>Log K values (M)</th>
<th>Log K values (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Partially purified CYP2B4</td>
<td>Av. value log K</td>
</tr>
<tr>
<td>7</td>
<td>-4.82, -4.94</td>
<td>-4.88</td>
</tr>
<tr>
<td>7.5</td>
<td>-4.30, -4.51</td>
<td>-4.41</td>
</tr>
<tr>
<td>8</td>
<td>-3.76, -4.00</td>
<td>-3.88</td>
</tr>
<tr>
<td>8.5</td>
<td>-3.27, -3.44</td>
<td>-3.36</td>
</tr>
<tr>
<td>9</td>
<td>-2.90, -3.09</td>
<td>-3.0</td>
</tr>
</tbody>
</table>

Table 3.2 The effect of pH on the calculated log K values for the titration of cyanide to partially purified and pure CYP2B4.
The binding of a selection of bases to oxidised partially purified CYP2B4 was undertaken in order to determine the base which would produce the greatest shift in wavelength. Partially purified CYP2B4 was used because of the very good isosbestic points observed on the binding of cyanide compared to those obtained for the pure enzyme (fig 3.1(F)). The bases investigated included the azines, azoles and amines, with the azoles producing the greatest shift in wavelength followed by the azines and then the amines. The apparent binding constants for these bases were calculated from log-log plots and are shown in table 3.3. The binding of the amines, trifluoroethylamine and hydroxylamine to CYP2B4 produced the smallest shift in wavelength with a correspondingly low log K value (table 4.12). The log K value for both trifluoroethylamine and hydroxylamine was deemed too low for an accurate study of the equilibria, thus these investigations were not carried out for these two bases. Of the azines
studied, pyridine was the only azine that produced a shift in wavelength (417-420), in addition to giving reproducible results for the calculation of log K. Although the shift in wavelength on the binding of pyridine to CYP2B4 was smaller than that exhibited by the azoles, it was thought appropriate to compare results obtained for oxidised CYP2B4 and pyridine, with those obtained for reduced CYP2B4 and pyridine (chapter 4). Due to the small shift in wavelength obtained on the binding of pyridine to CYP2B4, it is most likely that a greater error maybe incurred, especially when corrected for dilution, although binding is valid for up to 50%. The binding of N-acetylimidazole and imidazole to oxidised P450 produced the greatest shift in wavelength (table 3.1), but only N-acetylimidazole produced good isosbestic points (fig 3.4). The binding of this base to CYP2B4, like all the others was instantaneous with no further shift in wavelength observed on standing with time. Furthermore, the calculated average log K values (table 3.3) for both pyridine and N-acetylimidazole (N-AcIm) were found to be pH-independent (fig 3.5-6).
Fig 3.4 A spectrum produced on the binding of N-acetylimidazole to oxidised CYP2B4 at pH 8.5, clearly showing isosbestic points.

<table>
<thead>
<tr>
<th>Compound / pH</th>
<th>Binding constant values (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>31</td>
</tr>
<tr>
<td>N-acetylimidazole</td>
<td>562</td>
</tr>
</tbody>
</table>

Table 3.3 A table showing the apparent binding constants for the binding of pyridine and N-acetylimidazole to oxidised CYP2B4.
Fig 3.5 Log-log plots of N-acetylimidazole concentration against $\Delta A$ using a partially purified sample of oxidised CYP2B4 at pH 6.5 (o) and pH 8.5 (+).
Fig 3.6 Log-log plots of pyridine concentration against ΔA using a partially purified sample of oxidised CYP2B4 at pH 6.5 (o) and pH 8.5 (+).
3.4.3 Action of hydrogen peroxide on CYP2B4.

It is known that hydrogen peroxide can destroy cytochrome P450 (Guengerich 1990), thus in order to study the effect of hydrogen peroxide on CYP2B4, it was necessary to choose a concentration of hydrogen peroxide that would allow the spectrum to be studied over a suitable period of time without the rapid degradation of the enzyme. In order to achieve these conditions, a final concentration of 15 mM hydrogen peroxide was chosen which gave a half-life of 12 minutes. It can be seen from the spectrum that hydrogen peroxide completely degrades CYP2B4 without the production of a spectral intermediate or a shift in wavelength (fig 3.7. The kinetics of this degradation were found to be of a first order (value of \( A_\infty \) was extrapolated) at both pH 6.5 and 7.5 over at least 1 half-life (fig 3.8). The half-life for this degradation at these pHs was found to be 12 minutes for both, and shows that the degradation of CYP2B4 by hydrogen peroxide is pH-independent.

Fig 3.7 A spectrum showing the effect of 15 mM hydrogen peroxide concentration on oxidised pure CYP2B4 at pH 7.5, over a time period of 40 minutes.
3.4.4 Effect of hydrogen peroxide concentration.

The effect of hydrogen peroxide concentration on the degradation of CYP2B4 was carried out at two temperatures, 25 °C and 37 °C, using partially purified samples of CYP2B4. A few experiments were also carried out using pure CYP2B4 for comparison purposes with reactions involving lauric acid. The presence of the detergent, tergitol in the partially purified samples had little effect on the rate of degradation at both temperatures (table 3.4). The pseudo first order rate constant (k₁) at a given hydrogen peroxide concentration (1 M) was derived from the following equation $k_1 = 0.7/t_{1/2}$, (0.7 conversion factor) with the second order rate constant (k₂) calculated from the equation $k_2 = k_1/[H_2O_2]$. It can be concluded from the results in table 3.4 that the value of log k₂ at 25 °C was 0.2 ± 0.2, whilst at 37 °C this value was 0.5 ± 0.1 and was the same for both pure and partially purified samples and independent of whether tergitol was absent or present.
<table>
<thead>
<tr>
<th>Temperature</th>
<th>Hydrogen peroxide Concentration (mM)</th>
<th>Tergitol present</th>
<th>t 1/2 (minutes)</th>
<th>Calculated rate constant (k₁) min⁻¹</th>
<th>k₂ M⁻¹ min⁻¹</th>
<th>Log k₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C (partially purified)</td>
<td>15</td>
<td>yes</td>
<td>36</td>
<td>0.02</td>
<td>1.3</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>yes</td>
<td>25</td>
<td>0.03</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>yes</td>
<td>14</td>
<td>0.05</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>yes</td>
<td>8</td>
<td>0.09</td>
<td>1.5</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>no</td>
<td>38</td>
<td>0.02</td>
<td>1.3</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>no</td>
<td>16</td>
<td>0.04</td>
<td>0.8</td>
<td>-0.1</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>no</td>
<td>7</td>
<td>0.10</td>
<td>0.67</td>
<td>-0.2</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>no</td>
<td>4</td>
<td>0.175</td>
<td>0.58</td>
<td>-0.2</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>no</td>
<td>2</td>
<td>0.35</td>
<td>0.58</td>
<td>-0.2</td>
</tr>
<tr>
<td>37 °C (partially purified)</td>
<td>15</td>
<td>no</td>
<td>12</td>
<td>0.06</td>
<td>4.0</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>no</td>
<td>8</td>
<td>0.09</td>
<td>3.6</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>no</td>
<td>6</td>
<td>0.12</td>
<td>2.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>yes</td>
<td>25</td>
<td>0.03</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>yes</td>
<td>12</td>
<td>0.06</td>
<td>2.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>yes</td>
<td>8</td>
<td>0.09</td>
<td>3.0</td>
<td>0.5</td>
</tr>
<tr>
<td>25°C (pure)</td>
<td>30</td>
<td>no</td>
<td>14</td>
<td>0.05</td>
<td>1.7</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>no</td>
<td>8</td>
<td>0.09</td>
<td>1.5</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 3.4: The effect of temperature and hydrogen peroxide concentration on the rate of degradation of oxidized partially purified CYP2B4. The table also shows the calculated rate constant (k₁) and (k₂) values for this degradation of CYP2B4.

### 3.4.5 Effect of substrates and ligands on the rate of degradation (t₁/₂) of CYP2B4 by hydrogen peroxide.

The rate of cytochrome P450 degradation by hydrogen peroxide in the presence of substrates which may protect against the action of hydrogen peroxide was investigated using potential substrates such as benzphetamine, guaiacol, lauric acid and pentoxyresorufin and the ligand pyridine. Pyridine and the substrate guaiacol were the only compounds to affect the rate of degradation. Normally the half-life of CYP2B4 in the presence of 15 mM hydrogen peroxide was found to be 12 minutes, however, in the presence of 10 mM pyridine this was increased to 30 minutes. In the presence of guaiacol, a substrate for cytochrome...
P450 peroxidase activity, the half-life increased from 12 to 30 minutes. The substrates benzphetamine and lauric acid at concentrations of 1 mM and 0.05 mM respectively, both had no significant effects on the rate of degradation. The effect of the substrate pentoxyresorufin on the rate of degradation could not be determined due to background interference.

3.4.6 Lauric acid hydroxylation.

Initial experiments were carried out using iodosobenzene to establish a concentration of iodosobenzene that would produce a convenient rate of reaction (table 3.5). High concentrations of iodosobenzene (5 mM) caused a rapid decrease in absorbance at 417 nm (table 3.5). As a consequence, it was decided to use a concentration of 0.1 mM to investigate the hydroxylation of lauric acid by the ferryl intermediate.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Iodosobenzene concentration (mM)</th>
<th>Apparent half-life at 417 nm (minutes)</th>
<th>Time taken for peak to shift to 414 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C</td>
<td>0.10</td>
<td>(a) 4-6, (b) 8</td>
<td>&lt; 1 minute</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>7 (b)</td>
<td>&lt; 1 minute</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>6 (b)</td>
<td>&lt; 1 minute</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>&lt; 30 seconds (b)</td>
<td>&lt; 30 seconds</td>
</tr>
<tr>
<td>37 °C</td>
<td>0.01</td>
<td>20 (b)</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>6-8 (b)</td>
<td>&lt; 1 minute</td>
</tr>
</tbody>
</table>

Table 3.5: The action of iodosobenzene on cytochrome P450; duplicate experiments were carried out using partially purified samples at pH 7.5.

(a) pure CYP2B4

(b) partially purified CYP2B4

The spectrum produced by the action of iodosobenzene on partially purified CYP2B4 showed that the initial rate of reaction was very rapid, but did not go
to completion (fig 3.9). This rapid initial rate of reaction was also repeated using a pure sample of CYP2B4 and produced the same results. The plot of change in absorbance vs time shows that this reaction was of a first order which is similar to the reaction of hydrogen peroxide with CYP2B4 (fig 3.10). The reaction of iodosobenzene on CYP2B4 caused a shift in wavelength from 417 to 414 nm and the production of a new weak band at 385-390 nm which corresponds to the ferryl intermediate (fig 3.11). The production of this ferryl intermediate and the first order kinetics of the rate of reaction had been previously shown by Blake and Coon in 1989. The addition of hydrogen peroxide (15 mM) 8 minutes after the addition of iodosobenzene produced no significant increase in the rate of degradation (fig 3.10). This indicated that the iodosobenzene was blocking the action of hydrogen peroxide.

Fig 3.9 A spectrum produced by the action of 0.1 mM iodosobenzene on pure CYP2B4.
Fig 3.10 A plot of ΔA against time for the action of (a) 15 mM hydrogen peroxide (•) (b) 0.1 mM iodosobenzene (○) and (c) 0.1 mM iodosobenzene then 15 mM hydrogen peroxide, 8 minutes after the addition of the iodosobenzene (+) using a partially purified sample of CYP2B4 at a temperature of 25 °C.

Fig 3.11 A spectrum showing the production of the ferryl intermediate by the action of iodosobenzene on pure CYP2B4. ▼ intermediate
3.4.7 Identification of the metabolites of lauric acid hydroxylation.

Peak identification ideally should be carried out using hydroxylaurate standards or a system which generates all three hydroxylaurate metabolites, namely, \( \omega \), (\( \omega-1 \)) and (\( \omega-2 \)) hydroxylaurate. Since neither were available, a combination of systems was used to produce the three hydroxylaurate metabolites. The systems used were metabolites produced by microsomal clofibrate-induced CYP4A1 in the presence of NADPH and the metabolites produced by the action of hydrogen peroxide on lauric acid using pure CYP2B4. Clofibrate-induced microsomes were used as the source of CYP4A1, since clofibrate is the prototype inducer of this cytochrome P450 isozyme with lauric acid as its model substrate. The results from this experiment are shown in table 3.6. It can be seen from these results, that clofibrate-induced microsomes when utilising NADPH and molecular oxygen produced \( \omega \) and (\( \omega-1 \)) hydroxymetabolites in the ratio 5:1. While purified CYP2B4 produced on reaction with hydrogen peroxide the hydroxymetabolites (\( \omega-1 \)) and (\( \omega-2 \)), in the ratio 3:1. Thus by combining the extracts from both systems a three metabolite mixture in the ratio 10:4:1 was produced. The metabolites produced by hydrogen peroxide with CYP2B4 were identified to be the (\( \omega-1 \)) and (\( \omega-2 \)) hydroxymetabolites.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( \omega ) (%)</th>
<th>rt</th>
<th>34' Values</th>
<th>Av. value</th>
<th>( \omega-1 ) (%)</th>
<th>rt</th>
<th>32' Values</th>
<th>Av. value</th>
<th>( \omega-2 ) (%)</th>
<th>rt</th>
<th>37' Values</th>
<th>Av. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clofibrate microsomes (a)</td>
<td>23.00, 22.90, 20.20</td>
<td>22.03</td>
<td>4.00, 6.00, 4.70</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure CYP2B4 (b)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>4.40, 4.30</td>
<td>1.70, 1.50</td>
<td>1.60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clofibrate and Pure</td>
<td>24.00, 23.80</td>
<td>23.90</td>
<td>9.60, 9.40</td>
<td>3.20, 2.40</td>
<td>2.80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.6: The metabolites produced by the hydroxylation of lauric acid by either (a) NADPH or (b) hydrogen peroxide. Incubations were carried out in duplicate with an incubation period of 5 minutes, at 37 °C. (rt = retention time, ° minutes)
3.4.8 The effect of incubation time on lauric acid hydroxylation.

The graphs in figures 3.12 and 3.13 show the effect incubation time has on (ω-1) and (ω-2) hydroxylation. It can be seen that an increase in the length of incubation time, resulted in an increase in the amount of (ω-1) and (ω-2) hydroxylaurate metabolites produced (table 3.7). This increase was apparent for up to 10 minutes. After 10 minutes there was no substantial increase in (ω-1) or (ω-2) hydroxylation. The reaction of hydrogen peroxide with CYP2B4, when followed spectrophotometrically showed the degradation of the cytochrome P450 (fig 3.7). Therefore, the levelling off of the production of the hydroxymetabolites after 10 minutes is due to the degradation of the enzyme.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>(ω-1) % values</th>
<th>(ω-1) Av. value</th>
<th>(ω-2) % values</th>
<th>(ω-2) Av. value</th>
<th>ratio (ω-1)/(ω-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1.1, 1.3, 1.4</td>
<td>1.3</td>
<td>0.7, 0.9, 0.7</td>
<td>0.8</td>
<td>1.6</td>
</tr>
<tr>
<td>1</td>
<td>2.0, 1.6, 1.1</td>
<td>1.6</td>
<td>1.0, 1.2, 1.1</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>2.7, 2.8, 2.0</td>
<td>2.5</td>
<td>1.3, 1.2, 1.1</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>3</td>
<td>4.0, 2.5, 2.2</td>
<td>2.9</td>
<td>1.6, 1.0, 1.3</td>
<td>1.3</td>
<td>2.2</td>
</tr>
<tr>
<td>4</td>
<td>2.7, 3.6, 3.2</td>
<td>3.2</td>
<td>1.3, 1.3, 1.0</td>
<td>1.2</td>
<td>2.7</td>
</tr>
<tr>
<td>5</td>
<td>4.4, 4.0, 3.7, 3.8, 3.1, 3.9</td>
<td>3.8</td>
<td>1.3, 1.5, 1.5, 1.2, 1.4, 1.5</td>
<td>1.4</td>
<td>2.7</td>
</tr>
<tr>
<td>10</td>
<td>4.7, 4.6, 3.8, 3.5, 3.6, 4.2</td>
<td>4.1</td>
<td>1.7, 1.4, 1.6, 1.6, 1.6</td>
<td>1.5</td>
<td>2.8</td>
</tr>
<tr>
<td>20</td>
<td>5.8, 3.5, 4.8, 3.3</td>
<td>4.4</td>
<td>1.8, 1.4, 1.7, 1.6</td>
<td>1.6</td>
<td>2.9</td>
</tr>
<tr>
<td>40</td>
<td>4.6, 4.4, 4.2, 4.0</td>
<td>4.3</td>
<td>1.7, 1.5, 1.3, 1.6</td>
<td>1.5</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Table 3.7 The effect of incubation time on the amount of (ω-1) and (ω-2) hydroxymetabolites formed during the hydroxylation of lauric acid by 15 mM hydrogen peroxide at pH 7.5 in the presence of 0.05 mM lauric acid and 1μM pure CYP2B4. All results are expressed as percentage of total lauric acid added.
Fig. 3.12 A graph showing the effect of incubation time on the production of the (ω-1) metabolite from lauric acid hydroxylation supported by 15 mM hydrogen peroxide at 37 °C, pH 7.5 in the presence of 0.05 mM lauric acid. The amount of metabolite formed is expressed as a percentage of the total lauric acid added.

Fig. 3.13 A graph showing the effect of incubation time on the production of the (ω-2) metabolite from lauric acid hydroxylation supported by 15 mM hydrogen peroxide at 37 °C, pH 7.5, in the presence of 0.05 mM lauric acid. The amount of metabolite formed is expressed as a percentage of the total lauric acid added.
When the ratio of yields of the products formed from the hydroxylation of lauric acid by hydrogen peroxide is plotted against time, and extrapolated back to time 0, a ratio of approximately 1:1 for the products is achieved (fig 3.14). Thus, the ratio of products is seen to increase from 1:1 to approximately 3:1 (ω-1:ω-2) (table 3.7), suggesting that the conformation of the enzyme may be being relaxed, probably due to the reaction of hydrogen peroxide at some other site.
3.4.9 The products of lauric acid hydroxylation supported by either NADPH, hydrogen peroxide, cumene hydroperoxide and iodosobenzene.

a) NADPH

Microsomes from various sources fortified with NADPH were shown to hydroxylate lauric acid, at the \( \omega \) and \((\omega-1)\) positions (table 3.8). Both control microsomes and microsomes derived from animals pretreated with different inducers were found to hydroxylate lauric acid, but to different extents. Control microsomes produced \( \omega \) and \((\omega-1)\) hydroxymetabolites in a ratio of 1:1, which is the same ratio reported by other authors (Bjorkhem and Danielsson 1970; Azerad et al 1990). While microsomal samples from clofibrate treated animals, which is rich in CYP4A1 produced the \( \omega \) and \((\omega-1)\) metabolites in the ratio 14:1, these values compare favourably with those cited in the literature, where the ratio between the two products have been quoted as high as 17:1 (Cajacob et al 1988). Microsomes derived from phenobarbital treated animals, hydroxylated lauric acid at the \( \omega \) and \((\omega-1)\) positions in the ratio 1:5, whilst purified CYP2B4 produced the metabolites in the ratio 1:2. Literature values show that phenobarbital-induced microsomes produced these metabolites in a ratio of 1:3 (Bjorkhem and Danielsson 1970; Azerad et al 1990 and Tanaka et al 1990), whilst pure CYP2B4 generated the metabolites in a ratio ranging from 1:4 to 1:8 (Cajacob et al 1988; Tanaka et al 1990). The variation in the ratio of products formed from lauric acid hydroxylation when clofibrate and phenobarbital induced microsomes were used, can be ascribed to the rate of reaction. CYP4A1 oxidises lauric acid much faster than CYP2B4 when the reaction is initiated by NADPH and molecular oxygen (Ortiz de Montellano et al 1992).
Oxygen donors

\[ \text{Enzyme source} \quad \begin{array}{c|c|c|c}
\hline
& \text{O}_2 + 2\text{H} \quad \omega : \omega-1 : \omega-2 & \text{H}_2\text{O}_2 \quad \omega : \omega-1 : \omega-2 & \text{CuOOH} \quad \omega : \omega-1 : \omega-2 \\
\hline
\text{Control Microsomes} & 1 : 1 : 0 & 0 : 5 : 1 & 1 : 7 : 0 \\
& (1 : 1 : 0) & (0 : 6 : 1) & (1 : 9 : 0) \\
\text{Clofibrate-induced Microsomes} & 14 : 1 : 0 & 0 : 0 : 0 & 1 : 3 : 0 \\
& (17 : 1 : 0) & & \\
\text{Phenobarbital-induced Microsomes} & 1 : 5 : 0 & 0 : 2 : 1 & - \\
& (1 : 3 : 0) & & \\
\text{Pure CYP2B4} & 1 : 2 : 0 & 0 : 2 : 1 & 1 : 7 : 0 \\
& (1 : 4 : 0) & & \\
& to (1 : 8 : 0) & & \\
\hline
\end{array} \\
\]  

Table 3.8 A table showing the ratio of products (\(\omega\), \(\omega-1\), \(\omega-2\)) formed from the hydroxylation of lauric acid using various oxygen donors. Results are expressed as a ratio of the metabolites with published values in parenthesis. The experiment was carried out in duplicate using a 10 minute incubation period.

b) Hydrogen peroxide versus iodosobenzene.

The hydroxylation of lauric acid with hydrogen peroxide and iodosobenzene was used as a test for any degradation occuring during the hydroxylation reaction. Hydrogen peroxide was found to support the hydroxylation of lauric acid when CYP2B4 in the microsomal or pure form, or control microsomes were used as the source of cytochrome P450. However, the products formed from this hydroxylation were the \((\omega-1)\) and \((\omega-2)\) hydroxymetabolites (table 3.8). When CYP2B4 was used as the enzyme source the products were formed in a 2:1 ratio, whilst, with control microsomes the ratio of the products was 5:1, which compares to the ratio (4:1) reported in the literature (Romano et al 1988).

Hydrogen peroxide was found not to support the hydroxylation of lauric acid when microsomes from clofibrate induced animals were used as the source of the enzyme, suggesting that with hydrogen peroxide as the enzyme source the
reaction is faster with CYP2B4 than with CYP4A1.

When iodosobenzene was used as the oxygen source with pure CYP2B4, no hydroxylation of lauric acid occurred, which is in contrast to results reported by other authors, where hydroxylation occurred only at the (ω-2) position (Gustafsson and Bergman 1976). The lack of hydroxylation could be attributed to the rapid degradation observed spectrophotometrically when iodosobenzene was added to CYP2B4. As previously stated the action of iodosobenzene on CYP2B4 produces a spectral intermediate at 380-390 nm. This ferryl intermediate was thought to be involved in the hydroxylation reaction, and the lack of hydroxylation when iodosobenzene was added at the start of the reaction suggests that the initial rapid degradation of the enzyme blocks the hydroxylation reaction. It was therefore decided to form the ferryl intermediate first and then add lauric acid after this formation (after approximately 8 minutes), however no hydroxylation of the lauric acid was observed.

c) Different oxygen donors

The use of the different oxygen donors was used as a test for determining whether the production of different ratios of products was linked to conformational change occurring in the active site or a change in the reaction mechanism. The use of other hydroperoxides as the oxygen source, namely cumene hydroperoxide also resulted in the hydroxylation of lauric acid, but only at the (ω-1) position, when control and clofibrate microsomes were used, with more hydroxylation occurring with the control microsomes. This result suggests that the ratio of products of lauric acid hydroxylation cannot be used to decide whether the intermediate in this reaction is neutral hydrogen peroxide or the ferryl intermediate. As a general rule it can be stated that CYP2B4 produces more (ω-1) hydroxyproduct than (ω-2) or ω whether oxygen, hydrogen peroxide
or cumene hydroperoxide are used as the oxygen source, whereas CYP4A1 produces the ω metabolite in greater amounts than the (ω-1) metabolite.

3.4.10 Effect of hydrogen peroxide concentration on lauric acid hydroxylation.

An increase in hydrogen peroxide concentration from 7.5 to 30 mM, led to a non-linear increase in both (ω-1) and (ω-2) hydroxylation (table 3.9; Fig 3.15). However, the ratio of the hydroxymetabolites for each hydrogen peroxide concentration used remained the same. These observations may be attributed to an increase in the rate of degradation of the enzyme at the higher concentrations of hydrogen peroxide.

<table>
<thead>
<tr>
<th>Hydrogen peroxide concentration (mM)</th>
<th>(ω-1) values</th>
<th>Av. values</th>
<th>(ω-2) values</th>
<th>Av. values</th>
<th>Ratio of (ω-1):(ω-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>1.45, 0.97</td>
<td>1.21</td>
<td>0.97, 0.65</td>
<td>0.81</td>
<td>1.49</td>
</tr>
<tr>
<td>15</td>
<td>2.10, 1.13</td>
<td>1.62</td>
<td>1.13, 1.2</td>
<td>1.12</td>
<td>1.45</td>
</tr>
<tr>
<td>30</td>
<td>2.42, 2.26</td>
<td>2.34</td>
<td>1.45, 1.61</td>
<td>1.53</td>
<td>1.53</td>
</tr>
</tbody>
</table>

Table 3.9: The effect of varying hydrogen peroxide concentration on (ω-1) and (ω-2) hydroxylation of lauric acid at pH 7.5 with an incubation period of 1 minute. All results are expressed as nmol product produced / nmol P450 / minute.
3.4.11 The effect of pH on hydrogen peroxide supported hydroxylation of lauric acid.

In the presence of pure and microsomal samples of CYP2B4, hydrogen peroxide was found to support the hydroxylation of lauric acid. This hydroxylation was found to be pH independent for the (ω-1) and (ω-2) hydroxylation products over the pH range 7.5-9.0 (table 3.10). Furthermore, at pH below 7.0, there was a substantial increase in both (ω-1) and (ω-2) hydroxylation with all P450 samples used, although the ratio between the products formed remained constant (~2) at all the pHs investigated. Since lauric acid has a pK of between 4.5-5.0, it would be expected that the uncharged lauric acid (HLA⁺) would bind more strongly than the charged lauric acid (LA⁻). The increase in rate of hydroxylation and the absence of change in ratio can be explained by a higher binding of HLA⁺ without any significant changes in the relative orientations of the ω end of the enzyme and the Fe-H₂O₂ complex.
The effect of pH on the hydroxylation of lauric acid by hydrogen peroxide, using pure and microsomal samples of CYP2B4. All results are expressed as nmol product produced / nmol P450 / 10 minutes, (ND not determined).

### Table 3.10

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>22.6</td>
<td>10.6</td>
<td>5.7</td>
<td>3.0</td>
</tr>
<tr>
<td>7</td>
<td>16.7</td>
<td>6.9</td>
<td>4.4</td>
<td>2.0</td>
</tr>
<tr>
<td>7.5</td>
<td>4.6</td>
<td>2.2</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>4.3</td>
<td>2.2</td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>8.5</td>
<td>4.2</td>
<td>2.3</td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>2.4</td>
<td>1.9</td>
<td>0.6</td>
<td>ND</td>
</tr>
</tbody>
</table>

3.4.12 The effect of varying the lauric acid concentration on lauric acid hydroxylation.

Variation in $^{12}$C lauric acid concentration produced a concentration dependent effect on (ω-1) and (ω-2) hydroxylation, as reflected by the levels of the (ω-1) and (ω-2) hydroxylaurate product shown in table 3.11. As the concentration of lauric acid was increased, the rate of product formation (ω-1, ω-2), also increased linearly up to a lauric acid concentration of 0.1 mM. Throughout this increase in product formation, the ratio between the hydroxymetabolite products remained constant (Fig 3.16). This linear increase in (ω-1) and (ω-2) hydroxymetabolites is expected due to an increase in the occupancy of the substrate site.
Table 3.11 The effect of varying lauric acid concentration on the hydroxylation of lauric acid by hydrogen peroxide (15 mM) at pH 7.5. The incubation time was 1 minute. All results are expressed as nmol product produced / nmol P450 / minute.

<table>
<thead>
<tr>
<th>L.A. Concentration (mM)</th>
<th>(ω-1) values</th>
<th>Av. value</th>
<th>(ω-2) values</th>
<th>Av. value</th>
<th>Ratio of (ω-1)/(ω-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025</td>
<td>1.37, 1.94, 1.61</td>
<td>1.64</td>
<td>0.81, 1.13, 1.29</td>
<td>1.08</td>
<td>1.52</td>
</tr>
<tr>
<td>0.05</td>
<td>1.78, 3.87, 2.80</td>
<td>2.82</td>
<td>1.45, 1.94, 1.78</td>
<td>1.72</td>
<td>1.65</td>
</tr>
<tr>
<td>0.1</td>
<td>7.08, 5.48, 5.48</td>
<td>6.02</td>
<td>3.23, 4.19, 3.23</td>
<td>3.55</td>
<td>1.70</td>
</tr>
</tbody>
</table>

Fig 3.16 The effect of increasing lauric acid concentration on the rate of product formation, (● ω-1) and (○ ω-2) using pure CYP2B4 at 37 °C, pH 7.5 using 15 mM hydrogen peroxide.
3.4.13 The effect of tergitol on hydrogen peroxide supported hydroxylation of lauric acid.

Tergitol, the detergent used for the solubilisation of CYP2B4 in the purification of this enzyme, inhibited hydroxylation of lauric acid by hydrogen peroxide (table 3.12). However, when samples of cytochrome P450 which did not contain tergitol (desoaped) were used as the cytochrome P450 source, the production of the (ω-1) and (ω-2) hydroxylaurate metabolites was detected. In addition, to samples containing tergitol inhibiting hydroxylation, the same effect was observed when 0.1 % tergitol was added to "desoaped" CYP2B4. These results suggest that tergitol enters the binding site and inhibits lauric acid hydroxylation.

<table>
<thead>
<tr>
<th>P450 Sample</th>
<th>Hydroxymetabolites (ω-1)</th>
<th>Hydroxymetabolites (ω-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partially purified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(desoaped)</td>
<td>18.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Partially purified</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(contains tergitol)</td>
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<td></td>
</tr>
<tr>
<td>Partially purified</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(tergitol added)</td>
<td></td>
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</table>

Table 3.12: The effect of tergitol on the hydroxylation of lauric acid by 15 mM hydrogen peroxide at pH 7.5. Results are expressed as nmol product produced / nmol P450 / minute.
3.5 DISCUSSION.

The nature of the distal ligand (H₂O or HO⁻) in the resting ferric state of the enzyme was investigated by studying the pH-dependence of the equilibrium constants for the coordination of the Fe³⁺ ion in CYP2B4 using one anion, (CN⁻) and two uncharged bases (pyridine and N-acetylimidazole). The shift in the Soret band region from the initial wavelength of 417 nm during the spectrophotometric titration of ferric CYP2B4 with the bases (pyridine and N-acetylimidazole) and anion (CN⁻) falls in the order CN⁻ > N-acetylimidazole > pyridine with Soret bands at 436, 423 and 421 nm respectively (table 3.1). The subsequent accuracy of the isosbestic points provides an indicator of conformity with the assumed single equilibrium. The titration of the partially purified CYP2B4 enzyme with CN⁻ showed good to excellent isosbestic points (fig 3.1), together with reasonable log-log plots (fig 3.2) and a good linear increase of log $K_{app}$ with pH (fig 3.3). However, in contrast when pure CYP2B4 enzyme was used, broken isosbestic points (fig 3.1 (f)) and significantly poorer log-log plots (data not shown) were obtained, along with an obviously non-linear plot of log $K_{app}$ versus pH (fig 3.3), although the values of log $K_{app}$ at pH 8.0 and 8.5 for the pure enzyme agreed with those obtained for the partially purified enzyme at these pH's. This suggests the same intrinsic value of log $K_{app}$ but modified by the occurrence of additional pH-dependent changes at both higher and lower pH. The pK of HCN is known to be 9.2 and the observed linear increase in log $K_{app}$ with pH (below pK) means that the observed equilibrium corresponds to the coordination of free CN⁻ with the corrected pH-independent values of the true log K given in table 3.2.

The binding of pyridine and N-acetylimidazole to ferric partially purified CYP2B4 showed poorer isosbestic points which was probably caused by the smaller observed change in spectrum. It has been shown recently using a
combination of techniques, namely NMR and UV-Vis spectrophotometry, that substrate free P450cam will bind two molecules of pyridine (Banci et al 1994). The first of which has a value of K of $10^4$ M$^{-1}$, and is coordinated as a ligand to the metal, whilst the second pyridine molecule ($K \sim 10^3$ M$^{-1}$) binds in the hydrophobic pocket. This is also true for the binding of two molecules of imidazole and N-methylimidazole. Furthermore, substrate free cytochrome P450cam can also simultaneously bind one molecule of imidazole which is coordinated to the metal and one pyridine molecule which is bound in the hydrophobic pocket (Banci et al 1994). In addition, cytochrome P450cam can also form a ternary complex with both CN$^-$ and camphor, with the camphor molecule having little effect on either the spectrum or the affinity for CN$^-$. It can be assumed that the deviations observed in the binding of pyridine and N-acetylimidazole by CYP2B4 also reflect the binding of an additional molecule in the hydrophobic pocket subsequent to the coordination of the first to the Fe$^{3+}$ ion. The anomalies observed on the binding of the hydrophilic cyanide to partially purified and pure CYP2B4 maybe related to the amount of detergent present in the sample. The binding constants reported for cyanide, N-acetylimidazole and pyridine appear to be the first to be reported for the ferric state of any mammalian cytochrome P450. These pH-dependent studies show that cyanide binds as the anion (CN$^-$) while pyridine and N-acetylimidazole bind as the neutral base (tables 3.2-3.3).

As previously mentioned in the introduction controversy still surrounds the nature of the distal ligand in the low spin, 6 coordinated ferric state of cytochrome P450 as to whether it is water molecule or hydroxide ion (HO$^-$). Ignoring any pH-dependent configuration change, equilibria involving the substitution of coordinated water by anion or base would be expected to be pH-independent as is observed experimentally. However, it is difficult to see how such equilibria involving the substitution of coordinated HO$^-$ could be pH-
independent for both anions and bases, since this would require some special (presumably not merely coulombic) interaction which could differentiate \( \text{HO}^- \) from \( \text{CN}^- \), thiolates and probably most or all other anions. Until definite evidence for such a mechanism is produced, it seems logical to conclude that the low spin, 6 coordinate forms of ferric cytochrome P450 with a Soret band at 417 nm possesses water as the distal ligand.

The binding of many ligands and the calculation of their binding constants involving the ferric state have however been reported for the bacterial cytochrome P450\(_{\text{cam}}\). pH-independence has been reported for the binding constants for the uncharged dimethylsulphide (Me\(_2\)S) and for three thiolate anions RS\(^-\) over the pH range 7-9 (Sono and Dawson 1982a). The affinity of the thiocyanate anion (NCS\(^-\)) for cytochrome P450\(_{\text{cam}}\) also remains unchanged at both pH 7 and 8 although a lower value was reported at pH 6 and 9 (Sono et al 1986). However, the possibility of isomerisation between N and S-coordinated forms of the ligand prevents any definite conclusions being drawn. Binding or dissociation constants have also been reported for other ligands at a single pH, these ligands included CN\(^-\) (Sono and Dawson 1982a), pyridine (Banci et al 1994) and imidazole (Lipscomb 1980). Since the 2B4 isozyme shows no evidence for the multiple conformers observed with cytochrome P450\(_{\text{cam}}\) and CYP2A1, it would be expected that the observed isosbestic points and log-log plots for cytochrome P450\(_{\text{cam}}\) show deviations from the simple equilibrium at least as great as those observed for CYP2B4. However, no data have been reported on either the quality of the observed isosbestic points or on the analysis of the absorbance change.

The simple coordination of anions as well as bases by the Fe\(^{3+}\) ion has therefore been established for both cytochrome P450\(_{\text{cam}}\) (RS\(^-\) and Me\(_2\)S) and for CYP2B4 (CN\(^-\), pyridine and N-acetylimidazole). By contrast, peroxidases
only coordinate anions with the compulsory uptake of a proton (Sono et al 1986) according to equation 5, where P represents the protein including the binding site of the proton and water (OH₂).

\[ \text{P.Fe}^3(\text{OH}_2) + X^- + \text{H}^+ \leftrightarrow \text{+HP.Fe}^3.X^- + \text{H}_2\text{O} \quad (5) \]

This indicates that the Fe may be either 5 coordinate or 6 coordinate with water as the ligand. The binding constant for butylisocyanide by the Cys-ligated chloroperoxidase remains unchanged at pHs 3, 4.5 and 6, whilst those for all the anions studied, from strong acids such as HI to weak acids such as HCN, show a pH-dependence of the apparent binding constant which is related to its pK and indicates coordination of the undissociated HX or the equivalent coordination of X⁻ to the metal and the binding of H⁺ at some second site (Sono et al 1986). The resulting difference in the pH-independence of log K\text{app} for the coordination of cyanide (pK 9.2) between CYP2B4 (log K rising linearly with pH) and those reported for chloroperoxidase (independent of pH) indicate a requirement for the binding of the anion (CN⁻) and a proton (H⁺). The compulsory binding of an anion with the uptake of a proton and the related proton-coupled reduction of the Fe³⁺ ion by all peroxidases probably reflects the mechanism for "activating" hydrogen peroxide. In other words, conversion of the relatively unreactive hydrogen peroxide (pK 11.2) into the more reactive HO₂⁻ ion even at physiological pH by the co-operative coordination of HO₂⁻ to the metal and the binding of H⁺ at a second site. The availability of protein-free models for the proton-coupled reduction of Fe³⁺ porphyrins has clarified the role of coulombic interaction in such proton-coupled equilibria.

The evidence of a water molecule as the sixth ligand provided the basis for analysing the kinetics, including the pH-dependent reaction of cytochrome P450 with hydrogen peroxide which eventually leads to bleaching or haem degradation. When hydrogen peroxide on its own reacts with CYP2B4, complete
degradation or bleaching of the chromophore is observed with no detectable shift in the Soret band or accumulation of any intermediate (fig 3.7), which suggests that the first step in the reaction is the rate determining step. A prior reaction of cytochrome P450 with iodosobenzene to convert the ferric iron to the ferryl intermediate complex, termed compound I, inhibits the subsequent degradation of the chromophore by added hydrogen peroxide (fig 3.10). This strongly suggests that the ferryl complex is not an intermediate in the bleaching reaction, however, this is not conclusive proof, since the presence of iodosobenzene might somehow prevent the access of hydrogen peroxide to the active site. Furthermore, it is possible that the identification of the ferryl intermediate complex assumed here could turn out to be incorrect. The observed fall in absorbance at 417 nm with hydrogen peroxide follows first-order kinetics (fig 3.8), with the rate constants increasing approximately linearly with hydrogen peroxide concentration to give the second order rate constant ($k_2$) which was pH-independent at pH 6.5 and 7.5. In other words at pHs below the pK of hydrogen peroxide (11.2), the rate-determining step therefore obeys the rate equation - $d[Por]/dt = k_2[Por][H_2O_2]$, where Por represents the intact chromophore of cytochrome P450 and $k_2$ has the very low value of 0.6 M$^{-1}$ min$^{-1}$. Direct reaction of uncoordinated hydrogen peroxide with the porphyrin ring is unlikely both because several studies on porphyrin degradation by hydrogen peroxide have indicated the requirement for a redox-active porphyrin-chelated metal ion and because inhibition is observed by the known ligand pyridine (section 3.4.5). Since the distal ligand has been identified as water, the simplest explanation of the observed rate equation involves the initial formation according to equation 6 of the ferric iron complex with undissociated hydrogen peroxide as the ligand, probably at low concentration under equilibrium conditions.

$$H_2O_2 + [H_2O \Rightarrow Fe^3] \leftrightarrow H_2O + [H_2O_2 \Rightarrow Fe^3] \quad (6)$$
The fact that cytochrome P450 does not possess the "peroxidase" mechanism for converting hydrogen peroxide to the ferryl derivative through the proton-coupled coordination of HO$_2^-$ and that formation of the ferryl complex by another route using iodosobenzene actually inhibits further bleaching by hydrogen peroxide together strongly suggests that coordinated hydrogen peroxide is the key intermediate directly involved in attacking the porphyrin ring. These studies appear to be the first kinetic and mechanistic studies on the reaction of cytochrome P450 with hydrogen peroxide alone.

The bleaching of the porphyrin ring of cytochrome P450 has striking parallels with the bleaching by haem oxygenase. In comparing the reactions of haem oxygenase-bound haem with hydrogen peroxide and other peroxidases Wilks and Ortiz de Montellano (1993) found that m-chloroperbenzoic acid and bulky hydroperoxides such as butyl and cumyl hydroperoxides caused a shift in the Soret band which indicated the formation of a ferryl complex. Hydrogen peroxide on the other hand, produced a decrease in the Soret band without a corresponding shift in the wavelength and without the regeneration of the starting chromophore on addition of phenol. The sterically less hindered ethylhydroperoxide caused a small shift in wavelength with a partial recovery of the starting chromophore on the addition of phenol which was coupled with the formation of $\alpha$-meso-ethoxyhaem, which is indicative of some intermediate behaviour. It is therefore concluded from this work that a ferryl intermediate could not be the intermediate in the reaction with hydrogen peroxide and a mechanism involving coordinated HO$_2^-$ as the key hydroxylating intermediate with electrophilic addition of the distal oxygen atom (i.e HO$^+$) to the meso carbon atom has been proposed. However, no study of the pH-dependence of the rate was reported, no reason was given for assuming that hydrogen peroxide was coordinated as HO$_2^-$ rather than H$_2$O$_2$, and the proposed mechanism would leave O$_2^-$ as the unlikely ligand on the ferric ion. It is
therefore suggested that in haem oxygenase as in cytochrome P450, the ligand is probably present as the undissociated H₂O₂, which would both offer a far more electrophilic HO⁺ group for transfer and leave HO⁻ as a more reasonable ligand on the Fe³⁺ion, and thus able to pick up a further proton to reform the aquo complex, but this theory needs to be confirmed by studies on the pH-dependence of this reaction. The main point that can be concluded from this is, that in both haem oxygenase and cytochrome P450 the mechanistic pathway for bleaching by hydrogen peroxide involves a complex of iron and a two oxygen unit and apparently avoids any complex involving an iron and one oxygen unit or ferryl intermediate. This requirement must partly reflect the steric constraints of a reaction which involves the transfer of a group or atom and would require some intermediate which physically spans the distance between the "activating" Fe atom and the receiving carbon atom. Hydroxylation of an added substrate would clearly not be subject to such steric constraints. The use of the two reagents iodosobenzene and hydrogen peroxide therefore offers the opportunity to test whether the hydroxylation of a substrate such as lauric acid involves the reaction with an iron and one oxygen unit or iron and a two oxygen unit intermediate.

The cytochrome P450 catalysed hydroxylation of lauric acid can produce the ω, ω-1 and ω-2 isomers (table 3.8). The reaction occurs against a background of modification and eventual degradation of the haem enzyme by hydrogen peroxide (fig 3.7). The cumulative yields of products (ω-1, ω-2) levels off at approximately 6% of the total available lauric acid, after approximately 20 minutes as the enzyme becomes inactivated (fig 3.12-13). The ratio of the products changes with time from an extrapolated value of approximately 1.1 at t=0 to approximately 3:1 towards the end of the reaction (fig 3.14). This is the first case of lauric acid hydroxylation by hydrogen peroxide with any pure enzyme. The hydroxylation of lauric acid by four different potential oxygen
donors (O₂ and NADPH, hydrogen peroxide, iodosobenzene and cumene hydroperoxide) and the ratio of products formed is shown in table 3.8. The results show that CYP2B4 can catalyse the hydroxylation of lauric acid by hydrogen peroxide, cumene hydroperoxide as well as by molecular oxygen and NADPH, and that CYP2B4 can produce all three isomers in ratios which vary both with the nature of the oxygen donor and with time (table 3.8). Perhaps the most interesting fact is that no hydroxylation products could be detected when iodosobenzene was used as the oxygen donor, when it was added at the beginning of the reaction or after approximately 8 minutes, when the formation of the ferryl complex was complete.

The changes in the product ratios proposes a pertinent question as to whether these changes indicate a change in the reaction mechanism or merely a change in the structural constraints around the active site. This has been well studied in the case of the soluble methane monooxygenase (MMO) which, like cytochrome P450, can catalyse the hydroxylation of alkanes (including methane) using either molecular oxygen with NADH and a reductase and usually a third protein called component B, or hydrogen peroxide, termed systems I and II respectively. It has, however, a more complex (αβγ)₂ structure containing two μ-oxo bridged di-iron centres as the active site. Both systems I and II cause the same extent of chiral inversion during the hydroxylation of chiral 1-[¹H, ²H, ³H]-ethane, which was taken as evidence that "the nature of the chemistry of the reactions catalysed by systems I and II is the same". Increasing differences in the ratios of isomers produced were, however, observed with increasing size of the alkane (αω-1 ratios of approximately 0.7 and 13.3 for hexane with systems I and II respectively), which may be attributed to conformational changes at the reduced level in the reaction involving molecular oxygen. The variation observed in the product ratios with time but fixed oxygen donor (fig 3.14) can hardly be ascribed to a change in mechanism, while the variation with oxygen donor
(table 3.8) is no greater than the differences observed between systems I and II of methane monoxygenase. Different families of enzymes (CYP4A1, CYP2B4 and CYP2E1) also produce a different ratio of products with the same substrate, namely lauric acid (Hrycay et al 1976; Romano et al 1988), and testosterone (Sonderfan et al 1987) and the same oxygen donor (O2 and NADPH). It can therefore be concluded that such differences in product ratios cannot be used as evidence for differences in the nature of the key hydroxylating intermediate.

The yield of product is shown to increase roughly linearly with an increase in lauric acid concentration (fig 3.16), but falls off with increased hydrogen peroxide concentration (fig 3.15), this suggests that the rate of enzyme inactivation shows a greater than linear dependence on hydrogen peroxide concentration. The rate of hydroxylation is pH independent over the pH range 7.5-9.0, but increases below pH 7.5 (table 3.10), whilst the product ratio remains unchanged throughout. Since the pK of lauric acid is expected to be approximately 5.0, and the increase in rate at lower pHs can probably be ascribed to a higher degree of occupancy of the hydrophobic substrate-binding cavity by the undissociated acid compared to the dissociated anion, without any significant change in structure or mechanism around the active site. The basic reaction rate at pH 7.5-9.0 can therefore be described as pH independent and shows roughly a linear dependence of lauric acid concentration and would show a linear dependence on hydrogen peroxide concentration if the degradation of the enzyme could be suppressed. Interpretation of the kinetic data indicates that the reaction between lauric acid and hydrogen peroxide occurs via the anionic form of lauric acid (LA−) and undissociated hydrogen peroxide (H2O2) and not between undissociated lauric acid (HLA°) and hydrogen peroxide in its anionic form (HO2−). The fact that no spectral intermediate could be seen in the UV-Vis spectrum either during the slower reaction of hydrogen peroxide alone (fig 3.7) (i.e bleaching) or during the faster reaction in the presence of lauric acid
provides good evidence for assuming that anionic lauric acid (LA\textsuperscript{−}) and at lower pH undissociated lauric acid (HLA\textsuperscript{+}) reacts directly with undissociated hydrogen peroxide. This is supported by the failure of the ferryl intermediate produced from the reaction of iodosobenzene with CYP2B4 to react with lauric acid. The only other study of the pH dependence of a cytochrome P450 catalysed reaction involving hydrogen peroxide appears to be the oxidation of aminopyrine by hydrogen peroxide catalysed by CYP2B4 (Nordblom et al. 1976). The results observed a gradual increase in the rate of reaction with an increase in pH. From these results it was postulated that the HO\textsubscript{2}\textsuperscript{−} species was involved, with an increase in pH causing an increase in the rate of reaction. However, the rate of reaction only increased by a mere factor of 2 for the pH range 6.0-8.0 and not by the expected factor of 10\textsuperscript{2}. Their results do in fact provide good evidence for a reaction involving the undissociated hydrogen peroxide and not the HO\textsubscript{2}\textsuperscript{−} species.

CONCLUSION.

The pH-independent binding observed when the two neutral bases pyridine and N-acetylimidazole in association with the corrected log K values for the binding of the anion cyanide (CN\textsuperscript{−}) to CYP2B4, suggests that the sixth ligand present in the resting state of the enzyme is the water molecule (FeOH\textsubscript{2}) and not the hydroxide ion. The results in this chapter on the role of a peroxide complex in both lauric acid hydroxylation and haem degradation by CYP2B4, confirm and extend the results of Coon (CYP2B4 catalysed reaction of cyclohexane), Akhtar and co-workers (reactions involving 17\alpha-hydroxyandrogen and cytochrome P450\textsubscript{17\alpha}) on other cytochrome P450 catalysed reactions. The results also provide a natural explanation for the apparently close relationship between reactions 1-3

\begin{align*}
O_2 + 2H + RH &\rightleftharpoons \text{ROH} + H_2O (1); \\
O_2 + 2H &\rightleftharpoons H_2O_2 (2); \\
H_2O_2 + RH &\rightleftharpoons \text{ROH} + H_2O (3)
\end{align*}
and bleaching, and also demonstrate a common denominator in at least the initial step of haem degradation between cytochrome P450 and haem oxygenase (with Cys and His as the proximal ligand respectively). It also demonstrates a further common denominator in the ability of hydrogen peroxide to hydroxylate alkanes when associated with a sufficiently strong acid such as a proton (H$_3$O$_2^+$ in HF) or certain Lewis acids such as Fe$^{3+}$.

The main aim of this chapter was to test whether the key intermediate involved in hydroxylating a "solubilised" alkane such as lauric acid is a complex of the iron (Fe) with a one oxygen ligand (i.e ferryl complex) or a two oxygen ligand (H$_2$O$_2$ or HO$_2^-$). In light of the described experimental results and arguments, including comparisons with relevant data on other enzymes, it is proposed here that the balance of evidence strongly favours, but cannot conclusively prove, the Fe$^{3+}$H$_2$O$_2$ complex as the key reactive intermediate.
CHAPTER 4

Coordination of ligands by the \( \text{Fe}^{2+} \) ion
4.1 INTRODUCTION

In the ferric state (Fe(III)), the spectrum of cytochrome P450 includes the presence of α and β bands, along with an absorption maximum of either 418 or 390 nm depending on whether the enzyme is in a low 6 coordinate or high 5 coordinate spin state. These two states of the P450 enzyme appear to exist in an equilibrium (Schenkman et al. 1981). The addition of carbon monoxide to reduced cytochrome P450 causes a remarkable change in the spectrum; the Soret band intensifies and moves from 418 to 450 nm, no α band appears and the β band is seen around 550 nm. This distinctive feature of a substantially red-shifted Soret band at 450 nm in the Fe(II)-carbon monoxide derivative, is in contrast to that seen in myoglobin at 430 nm, and is associated with the presence of Cys in its anionic form RS⁻ as the proximal ligand (Guengerich 1991). The binding of certain ligands, such as the isocyanide derivatives (Fe(II)-CNR) shows two bands in the Soret region, at approximately 430 and 455 nm (single band in myoglobin at 434 nm), associated with the presence of reversible pH-dependent states or conformers (Imai and Sato 1967a). These were later denoted I and II with bands at 455 and 430 nm respectively, with conformer I favoured by high pH (Tsubaki et al. 1989). These changes observed with ethyl isocyanide are distinct from the almost irreversible change of cytochrome P450 to cytochrome P420 (denatured form). In cytochrome P420, it is thought that a His residue has replaced Cys as the proximal ligand, which then behaves spectrally like myoglobin as a normal haemoprotein; reduced cytochrome P420 gives only one Soret peak between 428-432 nm on combination with ethyl isocyanide (Wells et al. 1992). Furthermore, pH has no effect on this spectrum, which indicates that the anomalous properties of cytochrome P450 disappear when it is converted to P420 (Wells et al. 1992).
Two banded spectra may also be produced by other ligands such as pyridine (Imai and Sato 1967a), other isocyanides (Jefcoate and Gaylor 1969a), phosphites, phosphonites and phosphinites (Dahl and Hodgson 1978a), but not by compounds such as CO (450 nm), CN\(^{-}\) (436 nm), O\(_2\) (419 nm) or imidazole (428 nm) (Imai and Sato 1967a-b). The binding of oxygen is known to be anomalous; it shows a Soret band at 419 nm rather than as expected at ~430 nm, and is close to that exhibited by myoglobin (418 nm), but very different from that of chloroperoxidase at 430 nm (Sono et al 1985) which possesses Cys (RS\(^{-}\)) as the proximal ligand. It therefore seems likely that P450-O\(_2\) should be assigned to conformer II, due to its Soret peak, in addition to the fact that in microsomal P450, oxygen reacts preferentially with conformer II of the ethyl isocyanide complex (Imai and Sato 1966b) and lowering the pH promotes reaction with oxygen in competition with carbon monoxide (Ichikawa et al 1967). The unusual properties of coordinated oxygen can be linked to the presence of a lone pair of electrons on the uncoordinated \(\alpha\) atom, which is associated with an unexpectedly high nucleophilicity on the donor atom, termed the \(\alpha\) effect as with a strong hydrogen bonding to the lone pair of electrons.

The proximal ligand in cytochrome P450 is known to be a Cys in its thiolate RS\(^{-}\) form (Guengerich 1991). However, it has been proposed by Kahl and co-workers (1976) that in the reduced form this thiolate ligand exists in an equilibrium with its undissociated thiol form (RSH), and that this equilibrium can be represented by the following equation (1), where X denotes the distal ligand (RNC, pyridine etc) and subscripts I and II the two conformers, H\(^{+}\) can either represent a free proton in solution or a proton transferred from some neighbouring site.

\[
[\text{RS}^- \rightarrow \text{Fe} \, (\text{II}) \, \leftarrow \, X] \, \text{I} \, + \, \text{H}^+ \, \leftrightarrow \, [\text{RSH}^- \rightarrow \text{Fe} \, (\text{II}) \, \leftarrow \, X] \, \text{II} \quad (1)
\]
Recent work (Norris et al submitted for publication) with the iron porphyrin model microperoxidase 8 and sulphur ligands, has shown that the thiol/thiolate proximal ligand is readily accessible trans to the His residue in the reduced state. To investigate whether as previously proposed that the ligand in conformer II is the undissociated thiol, and that conformer I contains thiolate as its proximal ligand, the technique of EXAFS (Extended X-ray Absorption Fine Structure) will be used to confirm the ligand present in each of the conformational states.

There are two main theories concerning the two conformation states present in the ferrous form of the enzyme. It is the aim of the work in this chapter to investigate the role of the two conformation states of cytochrome P450. It has been proposed that hydrophilic compounds can form a single spectrum consisting of conformer I or II while hydrophobic compounds formed the double Soret spectra (Imai and Sato 1967a). This theory seems illogical since phosphonites, which are hydrophilic in nature produce double peaked spectra (Dahl and Hodgson 1978a). The second theory suggested that π bonding was involved in the production of the double peaked spectrum, however N-octylamine which cannot π bond can produce spectra containing two peaks (section 4.4.1). Since a similar effect is seen for pyridine and isocyanides in producing a spectrum consisting of two peaks, this indicates that metal to π bonding plays little or no role in producing this spectrum.

Recent work carried out on the coordination of three families of nitrogen bases by both an Fe(III) porphyrin (Hamza and Pratt 1994a) and a Co(III) corrinoid (Hamza and Pratt 1994b) have shown that the equilibrium constants K for coordination increases with the basicity pK of the ligand according to the relationship log K = a.pK + b, with similar values for a for all three families but values of b falling in the order azoles>azines>amines. The value of log K
may be further increased in the case of hydrazine, hydroxylamine and pyridazine through operation of the so-called \( \alpha \) effect. In order to confirm that \( \pi \) bonding does not participate in producing this spectrum, a wider range of bases was chosen for study, which included amines which do not undergo \( \pi \) bonding and azoles and azines which are known to undergo \( \pi \) bonding. The nitrogen bases shown in Figures 4.1 and 4.2 will be used to test the effect on equilibrium (1), firstly, of increasing electron density on the Fe (by increasing the pK of the base) and secondly, of ligands which may model a key feature of coordinated O\(_2\), in other words, the strong hydrogen bonding to a distal group (His E7 in haemoglobin) which is known to enhance the binding of O\(_2\) to haemoglobin, myoglobin and protein-free Fe(II) porphyrins (Jameson and Ibers 1994). The \( \alpha \) effect, a term originally applied to enhanced nucleophilic reactivity observed when the donor atom is attached directly to another electronegative atom carrying one or more lone pairs of electrons (Edwards and Pearson 1962), will be investigated using compounds such as hydroxylamine, hydrazine and pyridazine which all can act as models for this aspect of coordinated oxygen.

The transfer of the second electron in the cytochrome P450 catalytic cycle has been proposed to occur via a proton relay system involving a distal Thr/Ser residue (Gerber and Sligar 1994). As mentioned previously differences exist between cytochrome P450 and thromboxane synthase in that thromboxane synthase lacks the Thr and Ser residues, this enables investigation into the possible role of the Thr/Ser residues in the binding of hydroxylamine and trifluoroethylamine and comparing the results to those obtained with CYP2B4. The binding of nitrogen bases to different isozymes of cytochrome P450 will also be investigated during this chapter in order to establish any differences in the ratio of conformers between the different families.
Fig 4.1 Six-membered azines.

- Pyridine
- Pyrazine
- Pyrimidine
- 4-Aminopyridine
- 4-Cyanopyridine
- 4-Methylpyridine
- Pyridazine
- 4-Dimethylaminopyridine
Figure 4.2: Five-membered azoles.

- Imidazole
- 1,2,4-Triazole
- 1,2,3-Triazole
- 4-Nitroimidazole
- 5-Chloro-N-Methylimidazole
- N-Methylimidazole
- N-Acetylimidazole
4.2 MATERIALS.

These were described in section 2.2 and 3.2 for substances such as sodium dithionite. Additional chemicals and suppliers not mentioned previously are listed below. All materials were obtained from the sources cited and were of at least analytical grade and were used as received.

**Aldrich Chemical Company. (Gillingham, Dorset, UK)**
Aminopropionitrile, 4-aminopyridine, bromoethylamine, 5-chloro-N-methylimidazole, 4-cyanopyridine, dimethylaminopyridine, hydrazine, hydroxylamine, methylvamine, nitroimidazole, N-octylamine, 4-picoline, pyrazine, pyridazine, pyrimidine, 1,2,3 triazole and trifluoroethylamine.

**Fisons Scientific Equipment (Loughborough, Leicestershire, UK)**
Acetonitrile.

**Sigma Chemical Company. (Poole, Dorset, UK)**
Aminoacetonitrile, imidazole, methylimidazole and 1,2,4 triazole.
4.3 METHODS

Preparation of microsomal, partially purified and pure CYP2B4.

The samples of cytochrome P450 used for the experiments in this chapter were prepared as previously described in section 2.3.3.

4.3.1 Spectrophotometric assays.

Total protein content.

Total protein content of the cytochrome P450 samples was measured by the method of Lowry et al (1951) as previously described in section 2.3.10.

Total cytochrome P450 content.

The total amount of cytochrome P450 in the microsomal, partially purified and pure CYP2B4 samples were measured by the method of Omura and Sato (1964a) as previously described in section 2.3.10.
4.3.2 The effect of pH on the binding of azines, azoles and amines to reduced P450.

All investigations were carried out on a Uvikon 860 dual beam spectrophotometer.

The effect of pH on the binding of nitrogen containing compounds to cytochrome P450 was carried out using microsomal suspensions. The microsomes were diluted in 0.2 M potassium phosphate buffer at the stated pH, so that the final concentration of cytochrome P450 in the sample cuvette was 1.0 μM at a temperature of 25 °C.

The microsomal suspension was reduced by the addition of a few mgs of sodium dithionite, and subsequently divided into two 1.5 ml cuvettes and a baseline recorded. The compound under investigation was then added to the sample cuvette, so that the final concentration in the cuvette was 50 mM, except in the case of azoles, where lower concentrations were used (1 - 10 mM). A spectrum was then recorded. This procedure was carried out at the following pHs; 6.0, 6.5, 7.0, 7.5 and 8.0, using pure cytochrome P450 as well as microsomes.

4.3.3 The effects of varying pH on the reduced P450 difference spectrum in the presence of azines.

The effects of varying pH on the difference spectrum of reduced P450 with azines was investigated using pyridine.
(1) pH change prior to pyridine binding.

Microsomes were diluted in 0.2 M potassium phosphate buffer, pH 6.5. The resulting suspension was reduced by the addition of a few mgs of sodium dithionite, divided equally between two 1.5 ml cuvettes and a baseline recorded. The suspensions in both the sample and reference cuvettes were transferred to individual beakers and the pH was adjusted to 7.0 by the addition of 0.2 M potassium phosphate buffer, pH 9.0 containing sodium dithionite in order to ensure that the sample remained reduced. To adjust the pH from the initial pH (6.5), to the final pH (7.0), accurately, the exact volume of buffer required had been previously calculated by titration, using a pH meter to monitor the change. When the suspension in the reference and sample cuvettes were at pH 7.0, the cuvettes were autozeroed and pyridine was added to the sample cuvette, so that the final concentration of the ligand was 50 mM. The spectrum was then recorded. This procedure was also repeated for the following pH changes:

\[
7.0 \Rightarrow 7.5 \\
7.0 \Rightarrow 6.5 \\
7.5 \Rightarrow 7.0
\]

Decreases in pH were obtained by the addition of 0.2 M potassium phosphate pH 3.0 containing sodium dithionite.

(2) pH change after pyridine binding.

The procedure outlined above was used for this experiment, with the exception of where pyridine was added to the sample cuvette and a spectrum was recorded before pH adjustment.
4.3.4 Determination of binding constants of nitrogen-containing compounds to reduced cytochrome P450.

The binding constants of azines (pyridine and pyridazine), azoles (5-chloro-N-methylimidazole) and amines (trifluoroethylamine and hydroxylamine) were investigated using the methodology described below:

The determination of the binding constant for pyridine was carried out using 0.2 M potassium phosphate buffer, pH 6.5 and a microsomal sample of cytochrome P450. Microsomes were diluted to give a final concentration of 1.0 μM. The diluted microsomal suspension was reduced with a few mgs of sodium dithionite, divided equally between two 1.5 ml cuvettes and then a baseline was recorded between 400 nm and 500 nm. Pyridine was subsequently added to the sample cuvette so that the final concentration in the cuvette of pyridine was 1.0 mM. The spectrum was then recorded. Further aliquots of pyridine were added to the same cuvette, so that the concentration in the cuvette was increased by 1.0 mM on each addition. The above procedure was also repeated using partially purified and pure cytochrome CYP2B4. The determination of the binding constant of pyridazine, 5-chloro-N-methylimidazole, imidazole, trifluoroethylamine and hydroxylamine were carried out using the previously described procedure for pyridine except that partially purified cytochrome CYP2B4 was used initially instead of microsomes, but the final concentration of cytochrome P450 remained the same (1.0 μM).
4.3.5 The effects of time and ligand concentration on the binding of nitrogen compounds to reduced P450.

These experiments were carried out using 0.2 M potassium phosphate buffer, pH 8.0. The compounds investigated included pyridine and pyridazine (azines), 4-nitroimidazole (azole) and N-octylamine (amine). The same methodology was used for all compounds and is outlined below.

Microsomes were diluted to a final concentration of 1.0 µM using the buffer stated above, this microsomal suspension was then reduced with sodium dithionite and the baseline recorded. The compound under investigation was added to the sample cuvette so that the final concentration in the sample cuvette was either 20, 50, or 100 mM for pyridine, 0.1 mM for 4-nitroimidazole and 0.5 mM for N-octylamine. The spectra were then recorded. After this had been carried out, the cuvette was allowed to stand at room temperature for 30 minutes, and the spectra re-recorded. Carbon monoxide was bubbled through the sample cuvette at a rate of 1 bubble per second to produce a CO-difference spectrum. Pyridazine was also used in this time course experiment, but was carried out at pHs 6.0, 7.0 and 8.0, using a final concentration of 37 mM pyridazine.

4.3.6 Binding of nitrogen containing compounds to different cytochrome P450 isozymes.

The binding of nitrogen containing compounds to reduced cytochrome P450 derived from clofibrate and isoniazid treated animals was carried out using pyridine, imidazole, trifluoroethylamine and hydroxylamine.
Microsomes were diluted in 0.2 M potassium phosphate buffer, pH 6.0, 7.0 and 8.0 to give a final concentration of 1.0 μM. The microsomal suspension was reduced with sodium dithionite, divided equally between two 1.5 ml cuvettes and then a baseline was recorded. The compound under investigation was subsequently added to the sample cuvette to give a final concentration of 50 mM, except in the case of imidazole where the final concentration was 10 mM. The spectra were then recorded.

4.3.7 Competition between carbon monoxide, pyridine and imidazole for the reduced cytochrome P450.

Competition experiments on the reduced cytochrome P450 were carried out using 0.2 M potassium phosphate buffer, pH 7.0.

Microsomes were diluted to a final concentration of 1.0 μM, reduced with a few mgs of sodium dithionite and then divided equally between two 1.5 ml cuvettes. A baseline was subsequently recorded. Carbon monoxide was bubbled through the sample cuvette (1 bubble per second) and the spectrum was re-recorded. Pyridine was then added to the sample cuvette, to give a final concentration of 50 mM in the cuvette and the spectrum was re-recorded. The procedure was repeated, but pyridine was added prior to carbon monoxide addition. This procedure was repeated using imidazole at concentrations of 1 mM and 10 mM with either pyridine or carbon monoxide added both before and after the imidazole addition.
4.3.8 Identification of the proximal ligand present in the two conformational states of cytochrome P450.

EXAFS.

EXAFS (extended X-ray absorption fine structure) analysis was carried out to investigate the nature of the proximal ligand involved in the binding of nitrogenous bases to cytochrome P450.

The binding of certain ligands to cytochrome P450 can produce spectra which represent conformers I and II. In order to investigate the nature of the proximal ligand involved in the formation of these different conformers, nitrogen bases which only produce a single conformer were used for these studies, namely 1,2,3 triazole (conformer II) and cyanopyridine (conformer I).

Initial investigations were carried out using a concentrated solution of partially purified cytochrome P450 in the presence of either ligand, but the signal produced by EXAFS was too weak to enable any calculations to be carried out.

In order to produce a detectable EXAFS signal, a solution of partially purified P450 was concentrated down using aquacide to 5 ml, the smallest volume possible. A 1 ml aliquot of this concentrate was then centrifuged at 100,000 g (45,000 rpm) for one hour, using a Ti 70 rotor in an ultracentrifuge. The resulting pellet was resuspended in 0.2 M potassium phosphate buffer, pH 6.0. The remaining concentrate was already in 0.2 M potassium phosphate buffer, pH 7.5. A variation in the pH was used to enable the formation of only one conformer when the appropriate ligand was bound to the reduced cytochrome P450, i.e cyanopyridine (conformer I), pH 7.5, 1,2,3 triazole (conformer II), pH 6.0.
A portion (1 ml) of the concentrate at pH 7.5, was centrifuged at 100,000 g for one hour. The resulting pellet was then transferred to an EXAFS cell (volume 125 μl) and rapidly frozen in liquid nitrogen. The frozen cell was attached to a cryostat maintained at -70 °C whilst EXAFS analysis was carried out over a 12 hour period. Whilst EXAFS analysis was being carried out on the initial ferric sample, a second 1 ml sample of the concentrate was reduced with a few mg's of sodium dithionite at pH 7.5. Cyanopyridine, was subsequently added to the concentrate, so that the final concentration was 10 mM. This solution was now centrifugated at 100,000 g for one hour. The resulting pellet was then transferred to another EXAFS cell and rapidly frozen in liquid nitrogen. The cell was left in liquid nitrogen until required.

The ligand that produced conformer II was prepared as outlined above, except that the ligand was added to 0.2 M potassium phosphate buffer, pH 6.0, to give a final concentration of 1 mM

**Denaturation check.**

Before EXAFS analysis could be carried out investigations were performed on the concentrate to establish whether denaturation had occurred during pellet preparation.

Cyanopyridine or triazole was added to a portion (200 μl) of the concentrate in the appropriate buffer at the concentration to be used during EXAFS analysis. The suspension was reduced, using sodium dithionite and centrifuged at 100,000g for one hour. The resulting pellet was resuspended in 2 ml of the appropriate buffer, and placed into a sample cuvette and reduced. Extra ligand was then added to this cuvette and the spectrum recorded. The reference cuvette contained an equal amount of respun reduced concentrate. Carbon monoxide was
then bubbled through the sample cuvette and a spectrum re-recorded

4.3.9 Investigation into the role of the Threonine residue.

4.3.9.1 Preparation of thromboxane synthase.

The methodology employed for the preparation of thromboxane synthase from human platelets was based on a procedure developed by Needleman et al 1976.

Human platelet-rich plasma from 20 pints of blood was centrifuged at 200 g in a Beckman J6 for 10 minutes in order to remove the red blood cells. The resulting supernatant was centrifuged at 10,000 g for 6 minutes in a Beckman J2-21 to precipitate out the platelets. The resulting platelet pellet was divided into three unit fractions and was stored frozen at -70°C. The pellet concentrate (1 unit) was thawed and suspended in 3 ml of 100 mM Tris-HCl buffer, pH 7.5. This suspension was shell frozen rapidly (in dry ice/acetone) and then rapidly thawed. Once thawed it was homogenised in a glass-glass Potter homogeniser on ice. This procedure was repeated 3-4 times to maximise breakage of the cells. The resulting homogenate was centrifuged at 5,000 g for 15 minutes in a Beckman J2-21 centrifuge to remove the cell debris. The resulting supernatant was centrifuged at 100,000 g for 1 hour in a Beckman ultracentrifuge. The resulting pellet was gently washed with 100 mM Tris-HCl buffer, pH 7.5 and resuspended in 0.5 mls of ice cold Tris-HCl buffer, pH 7.5 and homogenised. This procedure was repeated for all the units and stored at -70 °C until required.
4.3.9.2 Determination of the total protein and haem content.

The total protein content of the platelet microsomes was determined using the methodology of Lowry et al 1951. Microsomal haem content in the platelets was determined using the method of Paul et al 1953, employing the reduced minus oxidised difference spectrum in pyridine/sodium hydroxide. A baseline was recorded between 530 nm and 570 nm with a freshly prepared pyridine/0.3M sodium hydroxide (1:2 v/v) mixture in both the reference and sample cuvettes. Once recorded, a portion (50 µl) of sample was diluted in pyridine/sodium hydroxide mixture so that the final volume in the cuvette was 1ml. The solution was then reduced with a few mgs of sodium dithionite, and a difference spectrum was recorded against pyridine/sodium hydroxide. Haem content was calculated using ΔE_{555-543} of 19.5 mM.

4.3.9.3 Binding of ligands to reduced thromboxane synthase.

All binding experiments were carried out on a Kontron Uvikon 942 spectrophotometer. The platelet homogenate was diluted in 0.2 M potassium phosphate buffer, pH 7.0, so that the final protein concentration in the cuvette was 4 mg/ml. The resulting suspension was reduced with a few mgs of sodium dithionite and divided equally between two 0.5 ml quartz cuvettes. A baseline was recorded between 390 and 500 nm. Initial binding experiments were carried out using carbon monoxide, where after recording the baseline carbon monoxide was bubbled through the sample cuvette for 1 minute and the spectrum repeatedly scanned for up to 30 minutes (10 x 3 minute scans). This was carried out in order to establish whether time had any effect on the spectrum. Once carbon monoxide binding had been established, the binding of nitrogen bases such as, pyridine, hydroxylamine and trifluoroethylamine was investigated. This was carried out by the addition of the ligand to the reduced sample in the
sample cuvette so that the final concentration was 50 mM in the case of pyridine and trifluoroethylamine and 10 mM for hydroxylamine. An equal amount of buffer was added to the reference cuvette. The spectra were repeatedly recorded for up to 50 minutes (10 x 5 minute scans) to establish the effects of time on the spectrum profile. Once this had been carried out, carbon monoxide was bubbled through the sample cuvette in order to determine whether the compound under investigation possessed any denaturing effects.

4.3.10 Ligand binding at the ferric level.

The binding of cyanide and two neutral bases (pyridine and N-acetylimidazole) were previously carried out in section 3.3.2 in order to determine the nature of the sixth ligand. Investigations into the binding of other nitrogen bases belonging to the following three families, azines, azoles and amines were carried out using partially purified CYP2B4 at a concentration of 1 μM.

The binding of nitrogen containing compounds to oxidised cytochrome P450.

Investigations into the binding of nitrogen containing compounds to oxidised cytochrome P450 were carried out initially, using partially purified cytochrome P450 at a concentration of 1.0 μM. The same procedure was used for each family of nitrogen compounds, the only difference being in the final concentration of ligand used.

The binding of pyridine, cyanopyridine and pyridazine to ferric cytochrome was investigated using 0.2 M potassium phosphate buffer pH 7.5. After the baseline had been recorded, the P450 suspension was placed in the sample cuvette, autozeroed and the spectrum recorded. Pyridine was then added to the sample
cuvette, so that the final concentration was 50 mM. The spectrum was re-recorded. Similar experiments were carried out using cyanopyridine and pyridazine at final concentrations of 10 mM and 50 mM respectively. The binding of imidazole and N-acetylimidazole to ferric cytochrome P450 was investigated using the procedure described for the azines, except that, the final concentrations of imidazole and N-acetylimidazole were 200 mM and 100 mM, respectively. The binding of hydrazine, hydroxylamine, methylamine, trifluoroethylamine and aminoacetonitrile to ferric cytochrome P450 was investigated using the above procedure described for the azines. All the compounds were added to the sample cuvette at a final concentration of 50 mM with the exception of aminoacetonitrile which was added at a final concentration of 150 mM. The binding of methylamine and hydroxylamine to ferric cytochrome P450 was also investigated under nitrogen. Prior to the recording of the oxidised spectrum of P450, nitrogen was bubbled into the sample cuvette in order to obtain a deoxygenated atmosphere. After the spectrum was recorded the ligand under investigation was added to the cuvette using a syringe under nitrogen, then the spectrum was re-recorded. The sample cuvette was subsequently left for 20 minutes and the spectrum re-recorded, in order to determine the effect of time, on the oxidised spectrum of cytochrome P450 on ligand binding.

4.3.11 Determination of binding constant of nitrogen containing compounds at the ferric level.

Binding constants were determined for the following nitrogen containing compounds with ferric cytochrome P450, namely, pyridazine, imidazole, trifluoroethylamine and hydroxylamine. A general procedure was employed for these determinations as outlined below:-
Partially purified cytochrome P450 was diluted to give a final concentration of 1.0 μM. After the baseline had been recorded using buffer, the partially purified cytochrome P450 suspension was placed in the sample cuvette and the spectrum recorded. Various amounts of the ligand were then added to the sample cuvette, whilst equal volumes of buffer were added to the reference cuvette. Titration of ligand to oxidised cytochrome P450 was carried out at the following pHs 6.5, 7.5 and 8.5 for all the ligands stated above.

4.3.12 The effect of time and addition of extra ligand to the oxidised binding spectrum of P450.

The effect of time and addition of extra ligand was previously investigated using the azine pyridine and reduced CYP2B4 and was found to cause denaturation (section 4.3.5). In view of this it was decided to investigate the effect of time and extra ligand on ferric CYP2B4 using the azines (pyridine and cyanopyridine), azoles, (imidazole and N-acetylimidazole), and amines (aminoacetoniitrile, hydrazine, hydroxylamine, methylamine and trifluoroethylamine).

The same procedure was used for all the ligands under investigation. Partially purified cytochrome P450 was diluted to give a final concentration of 1.0 μM. A baseline was recorded using buffer, then an aliquot of the cytochrome P450 suspension was added to the sample cuvette. The spectrum of the oxidised P450 was subsequently recorded. The ligand under investigation was added to the sample cuvette whilst a corresponding volume of buffer was added to the reference cuvette, and then the spectrum was re-recorded. After the spectrum had been recorded, the sample cuvette was left at room temperature for 15-20 minutes, after which the spectrum was re-recorded. A further aliquot of ligand was then added to the sample cuvette in order to determine whether a further
wavelength shift could be detected.
4.4 RESULTS.

4.4.1 The effect of pH on the binding of nitrogen bases to reduced CYP2B4.

When azines are bound to reduced cytochrome P450 (table 4.1); it can be seen that pH has an affect on the ratio of the conformers that are formed. In most cases the addition of azines causes the formation of a single Soret banded spectrum, corresponding to one or other of the conformers. However, in other instances, the addition of azines causes the production of a double Soret banded spectrum, where the conformers appear to be in a pH-dependent equilibrium, with low pH (6.0) favouring the formation of conformer II, whilst a high pH (8.0) favoured the formation of conformer I (fig 4.3). Some azines such as dimethylaminopyridine appeared not to bind to reduced CYP2B4 at any of the pHs used. The binding of carbon monoxide immediately after the binding of the base produced a spectrum with only one peak at 450 nm, indicating that no denaturation had taken place. At all pHs investigated the binding of azoles to reduced CYP2B4, produced a spectrum showing a single peak (table 4.2). For most of the azoles this single peak was at a wavelength of 422-429 nm, however, the binding of 0.1 mM nitroimidazole caused a peak at the wavelength 443 nm which increased in intensity with time. From these results it appears that for the majority of the azoles used, when bound to reduced cytochrome these compounds tend to favour conformer II, with the exception of nitroimidazole which forms conformer I. For all the azoles investigated, equilibrium was established within the time of mixing with the exception of nitroimidazole. On standing at room temperature no further shift in wavelength was observed for the azoles with the exception of 5-Cl-N-methylimidazole where denaturation was observed.
Fig 4.3 Spectra produced on the binding of pyridine to dithionite-reduced microsomes at various pHs. a) pH 6.0, b) pH 7.0, c) pH 8.0.
<table>
<thead>
<tr>
<th>Compound</th>
<th>pKa</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethylaminopyridine</td>
<td>9.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Aminopyridine</td>
<td>9.1</td>
<td>422</td>
<td>424</td>
<td>425</td>
</tr>
<tr>
<td>4-Picoline</td>
<td>6.0</td>
<td>423*</td>
<td>446</td>
<td>422*</td>
</tr>
<tr>
<td>Pyridine</td>
<td>5.2</td>
<td>424*</td>
<td>445</td>
<td>424</td>
</tr>
<tr>
<td>Pyridazine</td>
<td>2.3</td>
<td>428</td>
<td>426</td>
<td>425</td>
</tr>
<tr>
<td>4-Cyanopyridine</td>
<td>1.9</td>
<td>424</td>
<td>443*</td>
<td>443</td>
</tr>
<tr>
<td>Pyrimidine</td>
<td>1.1</td>
<td>444</td>
<td>443</td>
<td>442</td>
</tr>
<tr>
<td>Pyrazine</td>
<td>0.4</td>
<td>423</td>
<td>444*</td>
<td>424</td>
</tr>
</tbody>
</table>

Table 4.1: The effect of pH on the conformers formed when azines bind to reduced CYP2B4. The base was added at a final concentration of 50 mM.

* major conformer present.
- no binding.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKa</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylimidazole</td>
<td>7.2</td>
<td>ND</td>
<td>429</td>
<td>ND</td>
</tr>
<tr>
<td>Imidazole</td>
<td>7.1</td>
<td>430</td>
<td>428</td>
<td>429</td>
</tr>
<tr>
<td>N-Acetylimidazole</td>
<td>3.6</td>
<td>ND</td>
<td>430</td>
<td>ND</td>
</tr>
<tr>
<td>1,2,4-Triazole</td>
<td>2.3</td>
<td>426</td>
<td>426</td>
<td>426</td>
</tr>
<tr>
<td>1,2,3-Triazole</td>
<td>2.1</td>
<td>422</td>
<td>422</td>
<td>423</td>
</tr>
<tr>
<td>Nitroimidazole</td>
<td>-0.1</td>
<td>445</td>
<td>443</td>
<td>443</td>
</tr>
</tbody>
</table>

Table 4.2: The effect of pH on conformer formation when azoles are bound to reduced CYP2B4. All bases were added at a final concentration of 10 mM with the exception of nitroimidazole which was added at 0.1 mM.

ND - not determined.
The binding of a majority of amines to reduced cytochrome P450 showed a single peak in the difference spectra between 418-431 nm or 442-444 nm (table 4.3). However, the binding of N-octylamine was very different with a two banded spectrum being produced at pH 7.0 and 8.0. The appearance of the double spectrum at pH 7.0 (peaks at 426 and 443 nm) only lasted for 40 seconds, with an increase in time producing a corresponding increase in the formation of the peak corresponding to conformer II (426). While at pH 8.0 the initial spectrum consisted of a single peak at 426 nm and with time (10 minutes) the spectrum at this pH consisted of two peaks at wavelengths 426 and 447 nm. This slow change from a single conformer to a spectrum representing a mixture of conformers indicates that a slow reaction is taking place. The binding of N-octylamine was carried out in three different solvents (water, methanol and DMSO) with the same spectra seen with each solvent. The binding of N-octylamine using water as the solvent produced good isosbestic points at pH 7.0 clearly showing the change from a mixture of conformers to a single conformer with time (fig 4.4). The binding of ammonia only produced a spectrum at higher pH while methylamine did not produce a spectrum. These results were attributed to the pH used being well below the pKa value. The effect of lowering the pH on the binding of bromoethylamine and hydroxylamine caused the intensity of the peak to be reduced.
Table 4.3: The effect of pH on the binding of amines to reduced cytochrome P450.

* wavelength changes with time (426), ** wavelength shifts from 426 at start to 447.
ND not determined, - no binding. ¹ indicates bases that exhibit α effect.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKa</th>
<th>6</th>
<th>6.5</th>
<th>7</th>
<th>7.5</th>
<th>8</th>
<th>8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Octylamine</td>
<td>10.65</td>
<td>427</td>
<td>428</td>
<td>426</td>
<td>443*</td>
<td>ND</td>
<td>425</td>
</tr>
<tr>
<td>Methylamine</td>
<td>10.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ammonia</td>
<td>9.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>434</td>
<td>431</td>
</tr>
<tr>
<td>Bromoethylamine</td>
<td>8.5</td>
<td>-</td>
<td>432</td>
<td>432</td>
<td>431</td>
<td>428</td>
<td>429</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>8.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>434</td>
<td>431</td>
<td>ND</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>6.0</td>
<td>418</td>
<td>420</td>
<td>418</td>
<td>420</td>
<td>420</td>
<td>ND</td>
</tr>
<tr>
<td>Trifluoroethylamine</td>
<td>5.7</td>
<td>442</td>
<td>442</td>
<td>443</td>
<td>442</td>
<td>443</td>
<td>ND</td>
</tr>
<tr>
<td>Aminoacetonitrile</td>
<td>5.3</td>
<td>443</td>
<td>444</td>
<td>443</td>
<td>442</td>
<td>443</td>
<td>442</td>
</tr>
</tbody>
</table>

Fig 4.4 A spectrum showing the slow change from conformer I to conformer II which occurs on the binding of N-octylamine to reduced CYP2B4 with time at pH 8.0.

a) conformer I   b) conformer II
4.4.2 The effect of varying pH on the difference spectrum produced by pyridine.

These present studies were undertaken to ascertain whether changing the pH in both directions (e.g., 7.0<=>6.5 and 7.0<=>7.5) after reduction was affected by the presence of pyridine added after or before change in pH. A change in the pH, either by increasing or decreasing the pH, produced on binding of pyridine a spectrum resembling that of the new pH, e.g., if the initial pH was 7.0, the spectrum would consist of a mixture of conformers, if the pH was now changed to pH 6.5, the spectrum would now resemble that of pH 6.5, with conformer II the major form present, although the intensity of the peaks would be reduced due to dilution (fig 4.5). Varying the pH after pyridine was bound to the reduced cytochrome P450 was found to produce similar spectra to what was expected, in that pyridine already bound to the cytochrome P450 did not block the change between conformers. In both cases the binding of pyridine was instantaneous with no further slow change in the ratio of conformers. From these results the addition of pyridine before or after pH change did not prevent the interconversion between the two conformers. This interconvertibility between the conformers was also found to be reversible, however reversibility was limited due to dilution.

4.4.3 Determination of a relationship between pKa and the binding of nitrogen bases at fixed pH.

A relationship was found to exist between pKa and the wavelength observed when nitrogen bases bind to reduced CYP2B4 at pH 7.0. For the majority of the bases studied, those with a high pKa favoured the formation of conformer II. While those bases with a low pKa favoured the formation of conformer I (table 4.4-6). However, in the case of pyridazine, its low pKa value suggests
Fig 4.5 Spectra showing the pH-dependent change in the pyridine difference spectra of reduced microsomal CYP2B4

a) i) pH 6.5  
   ii) pH 6.5 ⇒ 7.0

b) i) pH 7.0  
   ii) pH 7.0 ⇒ 6.5

c) i) pH 7.0  
   ii) pH 7.0 ⇒ 7.5

d) i) pH 7.5  
   ii) pH 7.5 ⇒ 7.0
that it should favour conformer I, however, our experimental data indicates that
conformer II was formed. The bases dimethylaminopyridine, methylamine and
ammonia did not appear to bind to reduced cytochrome P450 at the pH used,
which was indicated by the lack of detectable peaks due to the pH used being
below their pKa values. Pyridine, 4-picoline and N-octylamine produced a
mixture of conformers at pH 7.0, with conformer I being slightly more
favoured than conformer II (table 4.4 and 4.6). The mixture of conformers (I
and II) produced by N-octylamine at pH 7.0 was only observed for the initial
40 seconds, with the peak representing conformer II increasing with time and
reaching a maximum after two minutes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKa</th>
<th>conformer 1</th>
<th>conformer 2</th>
<th>Ratio A_I : A_{II}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethylaminopyridine</td>
<td>9.8</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
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<td>4-Aminopyridine</td>
<td>9.1</td>
<td>_</td>
<td>423</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td>4-Picoline</td>
<td>6</td>
<td>446</td>
<td>423</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridine</td>
<td>5.2</td>
<td>446</td>
<td>423</td>
<td>1.25</td>
</tr>
<tr>
<td>Pyridazine *</td>
<td>2.3</td>
<td>_</td>
<td>427</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>4-Cyanopyridine</td>
<td>1.9</td>
<td>443</td>
<td>_</td>
<td>&gt; 10</td>
</tr>
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<td>Pyrimidine</td>
<td>1.1</td>
<td>443</td>
<td>_</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>Pyrazine</td>
<td>0.4</td>
<td>443</td>
<td>_</td>
<td>&gt; 10</td>
</tr>
</tbody>
</table>

Table 4.4: Relationship between pKa and formation of conformers I and II with azines at
pH 7.0.
- no binding, * show α effect.
<table>
<thead>
<tr>
<th>Compound</th>
<th>pKa</th>
<th>conformer 1</th>
<th>conformer 2</th>
<th>Ratio $A_I : A_{II}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylimidazole</td>
<td>7.2</td>
<td></td>
<td>429</td>
<td>$\leq 0.1$</td>
</tr>
<tr>
<td>Imidazole</td>
<td>7.1</td>
<td></td>
<td>429</td>
<td>$\leq 0.1$</td>
</tr>
<tr>
<td>N-Acetylimidazole</td>
<td>3.6</td>
<td></td>
<td>430</td>
<td>$\leq 0.1$</td>
</tr>
<tr>
<td>1,2,4 Triazole</td>
<td>2.3</td>
<td></td>
<td>426</td>
<td>$\leq 0.1$</td>
</tr>
<tr>
<td>1,2,3 Triazole</td>
<td>1.2</td>
<td></td>
<td>422</td>
<td>$\leq 0.1$</td>
</tr>
<tr>
<td>4-Nitroimidazole</td>
<td>-0.1</td>
<td>446</td>
<td></td>
<td>$\geq 10$</td>
</tr>
</tbody>
</table>

Table 4.5: Relationship between pKa and the formation of conformers I and II with azoles at pH 7.0. - no binding observed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKa</th>
<th>conformer 1</th>
<th>conformer 2</th>
<th>Ratio $A_I : A_{II}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Octylamine</td>
<td>10.65</td>
<td></td>
<td>426-7</td>
<td>$\leq 0.1$</td>
</tr>
<tr>
<td>Methylamine</td>
<td>10.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonia</td>
<td>9.3</td>
<td></td>
<td></td>
<td>(&lt;0.1)</td>
</tr>
<tr>
<td>Bromomethylamine</td>
<td>8.5</td>
<td></td>
<td>428-32</td>
<td>$\leq 0.2$</td>
</tr>
<tr>
<td>Hydrazine $^1$</td>
<td>8.1</td>
<td></td>
<td></td>
<td>(&lt;0.1)</td>
</tr>
<tr>
<td>Hydroxylamine $^1$</td>
<td>6</td>
<td></td>
<td>419</td>
<td>$&lt; 0.1$</td>
</tr>
<tr>
<td>Trifluoroethylamine</td>
<td>5.7</td>
<td>442</td>
<td></td>
<td>$\geq 10$</td>
</tr>
<tr>
<td>Aminoacetonitrile</td>
<td>5.3</td>
<td>442</td>
<td></td>
<td>$\geq 10$</td>
</tr>
</tbody>
</table>

Table 4.6: Relationship between pKa and formation of conformers I and II by amines at pH 7.0.

- no binding, * binding only at higher pH (8.5-9.0), $^1$ bases that show α effect.
4.4.4 Determination of binding constants.

The apparent binding constants for a series of nitrogen bases were determined by the addition of small volumes of stock ligand to the sample cuvette, and equal amounts of buffer to the reference cuvette. The absorbance values observed on each ligand addition were ascertained. In all cases, binding appeared to be instantaneous and the average absorbance values from duplicate experiments were taken and used to construct log-log plots, from which the values of \( K \) were calculated (table 4.7). The curves produced in each case corresponded to the binding of one molecule of base per Fe (figs 4.6-8). The binding constant for pyridine was carried out using a microsomal sample of CYP2B4, because when pure CYP2B4 was used there was an increase in denaturation as shown by the increase in absorbance at 426 nm (fig 4.6). The calculated binding constants values for pyridazine, hydroxylamine and trifluoroethylamine were found to be pH independent as illustrated by table 4.7. The binding constant value for hydroxylamine was obtained by estimating the end point, due to a decrease in absorbance at the higher concentrations of ligand (fig 4.8).

<table>
<thead>
<tr>
<th>Compound / pH</th>
<th>Binding constant values (M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Pyridine</td>
<td>_</td>
</tr>
<tr>
<td>Pyridazine</td>
<td>13</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>_</td>
</tr>
<tr>
<td>Trifluoroethylamine</td>
<td>_</td>
</tr>
</tbody>
</table>

Table 4.7 A table showing the calculated binding constants of nitrogen bases to reduced CYP2B4 at various pHs. - not determined
Fig 4.6 A graph showing the increase in the mixture of conformers (+1; o I ) formed on the binding of pyridine to reduced CYP2B4 at pH 6.5.

Fig 4.7 A log-log plot of pyridazine concentration against ΔA using reduced partially purified CYP2B4 at pH 6.0 (+), 7.0 (o) and 8.0 (+).
Fig 4.8 A graph showing the initial increase in absorbance observed versus concentration of hydroxylamine added to a sample of reduced CYP2B4 at pH 7.0, and the decrease observed at higher concentrations of hydroxylamine.

Fig 4.9 A log-log plot of hydroxylamine concentration against $\Delta A$ at pH 7.0 (•) and pH 8.0 (o) using reduced partially purified CYP2B4.
4.4.5 The effects of concentration and time on the observed difference spectra at fixed pH.

Concentration

For the majority of azines, azoles and amines used in this study, variation in ligand concentration did not affect conformer formation as indicated by the observed difference spectra (tables 4.8). In the case of the azines, namely pyridine, pyrazine and 4-picoline which produced a mixture of conformers I and II on binding to reduced cytochrome P450, a variation in ligand concentration had no effect on the conformers formed, although at high concentrations of pyridine (100 mM) higher levels of conformer II was found to be formed. This was later attributed to denaturation, since when carbon monoxide was immediately bubbled through the cuvette, formation of a peak at 420 nm was revealed. In the case of the azoles, 4-nitroimidazole and N-acetylimidazole, binding spectra were only obtained at low concentrations of the ligand (0.1 and 5 mM respectively). Binding of 4-nitroimidazole at high concentrations (10 mM) was not observed due to its insolubility at these high concentrations, whilst for N-acetylimidazole high background interference was observed which was due to its intense colour at concentrations above 25 mM which may be responsible for the difficulty in obtaining a difference spectra.

Time.

Time course experiments have revealed that in the majority of cases, time has no effect on conformer formation when ligands such as N-acetylimidazole, methylimidazole, nitroimidazole, 1,2,4 triazole and trifluoroethylmine were bound to reduced cytochrome P450, i.e there is no change in type of conformer with respect to time and no denaturation as indicated by a lack of a peak at 420 nm
in the carbon monoxide spectrum. They all produced a single peak at 450 nm. However, this does not hold true for pyridine, pyridazine, 5-chloro-N-methylimidazole or N-octylamine. Pyridine was found to produce a peak at 420 nm which was later attributed to denaturation, since the peak was obtained without the addition of carbon monoxide. In the case of the other ligands mentioned, namely pyridazine and 5-chloro-N-methylimidazole, the bubbling of carbon monoxide through the sample cuvette produced two peaks in the difference spectrum at 421 and 450 nm. This was indicative of CYP2B4 denaturation over a period of time, even though the denaturation was only observed when carbon monoxide was present. In the case of pyridazine, low pH caused the denaturation of CYP2B4, whilst at pH 8.0, only a single peak at 450 nm was observed on the binding of carbon monoxide, indicating that low pH increases the rate of denaturation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>Concentration (mM)</th>
<th>Initial peak(s)</th>
<th>Time (minutes)</th>
<th>Final peak(s)</th>
<th>Peak(s) after carbon monoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>7</td>
<td>50</td>
<td>422 445*</td>
<td>0</td>
<td>422 446*</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>50</td>
<td>422 445*</td>
<td>30</td>
<td>422* 445</td>
<td>421 450*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>100</td>
<td>425* 446</td>
<td>30</td>
<td>421* 446</td>
<td>421* 450</td>
</tr>
<tr>
<td>Pyridazine</td>
<td>7</td>
<td>50</td>
<td>425</td>
<td>0</td>
<td>426</td>
<td>421 450</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>50</td>
<td>426</td>
<td>20</td>
<td>426</td>
<td>421 450</td>
</tr>
<tr>
<td>5-Cl-N-methylimidazole</td>
<td>7.0</td>
<td>50</td>
<td>424</td>
<td>0</td>
<td>424</td>
<td>421 449</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>50</td>
<td>424</td>
<td>10</td>
<td>424</td>
<td>422 449</td>
</tr>
<tr>
<td>N-octylamine</td>
<td>7.0</td>
<td>0.5</td>
<td>426 446</td>
<td>0</td>
<td>426 446</td>
<td>449</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>0.5</td>
<td>426 446</td>
<td>5</td>
<td>426</td>
<td>449</td>
</tr>
</tbody>
</table>

Table 4.8.: The effect of time on the ratio of conformers formed when nitrogen bases bind to reduced cytochrome P450.

* major conformer present
4.4.6 Binding of nitrogen bases to different cytochrome P450 families.

CYP4A1 and CYP2E1, which are induced by clofibrate and isoniazid respectively were chosen for these experiments due to their availability.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Isozyme</th>
<th>Wavelengths observed at pH 6.0 (ratio A&lt;sub&gt;i&lt;/sub&gt;/A&lt;sub&gt;II&lt;/sub&gt;)</th>
<th>Wavelengths observed at pH 7.0 (ratio A&lt;sub&gt;i&lt;/sub&gt;/A&lt;sub&gt;II&lt;/sub&gt;)</th>
<th>Wavelengths observed at pH 8.0 (ratio A&lt;sub&gt;i&lt;/sub&gt;/A&lt;sub&gt;II&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>4A1</td>
<td>425* 447 (&lt;0.2)</td>
<td>425 446 (1.0)</td>
<td>424 447* (2.2)</td>
</tr>
<tr>
<td></td>
<td>2B4</td>
<td>425* 449 (&lt;0.2)</td>
<td>424 447 (1.3)</td>
<td>424 446* (1.5)</td>
</tr>
<tr>
<td></td>
<td>2E1</td>
<td>425* 449 (&lt;0.2)</td>
<td>424 447 (0.7)</td>
<td>424 446* (1.8)</td>
</tr>
<tr>
<td>Imidazole</td>
<td>4A1</td>
<td>429 (&lt;0.1)</td>
<td>428 (&lt;0.1)</td>
<td>429 (&lt;0.1)</td>
</tr>
<tr>
<td></td>
<td>2B4</td>
<td>430 (&lt;0.1)</td>
<td>428 (&lt;0.1)</td>
<td>429 (&lt;0.1)</td>
</tr>
<tr>
<td></td>
<td>2E1</td>
<td>428 (&lt;0.1)</td>
<td>427 (&lt;0.1)</td>
<td>427 (&lt;0.1)</td>
</tr>
<tr>
<td>Trifluoroethylamine</td>
<td>4A1</td>
<td>444 (&gt;10)</td>
<td>443 (&gt;10)</td>
<td>443 (&gt;10)</td>
</tr>
<tr>
<td></td>
<td>2B4</td>
<td>442 (&gt;10)</td>
<td>442 (&gt;10)</td>
<td>442 (&gt;10)</td>
</tr>
<tr>
<td></td>
<td>2E1</td>
<td>443 (&gt;10)</td>
<td>444 (&gt;10)</td>
<td>443 (&gt;10)</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>4A1</td>
<td>421 (&lt;0.1)</td>
<td>419 (&lt;0.1)</td>
<td>421 (&lt;0.1)</td>
</tr>
<tr>
<td></td>
<td>2B4</td>
<td>418 (&lt;0.1)</td>
<td>418 (&lt;0.1)</td>
<td>420 (&lt;0.1)</td>
</tr>
<tr>
<td></td>
<td>2E1</td>
<td>421 (&lt;0.1)</td>
<td>420 (&lt;0.1)</td>
<td>422 (&lt;0.1)</td>
</tr>
</tbody>
</table>

Table 4.9: The binding of nitrogen bases at a concentration of 50 mM to different cytochrome P450 families at the ferrous level.

* major conformer

Similar difference spectra were obtained for the binding of nitrogen bases to reduced cytochrome P450 samples derived from phenobarbital (2B4), clofibrate (4A1) and isoniazid (2E1) treated animals at pH 7.0 (table 4.9). Of all the bases studied, pyridine was the only base to produce a mixture of conformers on binding to the different cytochrome P450 families. Furthermore, when pyridine was bound to all the families under investigation, at low pH, formation of conformer II was favoured, whilst at high pH, conformer I was favoured. In the case of hydroxylamine, for all cytochrome P450 families studied, a decrease in pH was paralleled by a decrease in the intensity of the conformer II.
However, in the case of imidazole and trifluoroethylamine, this decrease was not observed. These two compounds produced peaks at 429 and 442 nm, respectively. The binding of all bases was instantaneous at the concentration used (50 mM).

4.4.7 Competition.

Carbon monoxide (CO) was found only to produce one peak at 450 nm when bound to reduced cytochrome P450 and this binding was not affected by a change in pH (6-8). When pyridine was bound to reduced cytochrome P450 a difference spectrum consisting of two peaks was observed. When this solution was bubbled with carbon monoxide only one peak at 450 nm was seen. This finding is similar to imidazole when it was bound to reduced cytochrome P450, a single peak was seen at 429 nm in the difference spectrum, but after carbon monoxide addition this single peak was shifted to 450 nm. These initial experiments suggests that cytochrome P450 has a higher affinity for CO than pyridine or imidazole. When imidazole was added prior to pyridine addition the spectrum obtained changed from a single peak spectrum to the characteristic two peaked spectrum of pyridine. Thus, it appears the affinity order of these ligands is:

\[ \text{CO > pyridine > imidazole} \]

4.4.8 Identification of the sixth ligand.

EXAFS.

The technique of EXAFS was attempted in order to determine the nature of the ligand, thiol or thiolate, involved in the binding of nitrogen bases to reduced CYP2B4. The nature of the ligand was investigated using various samples of
CYP2B4. Native ferric CYP2B4, ferrous CYP2B4 with the ligand cyanopyridine bound, which produces conformer I only and ferrous CYP2B4 with the ligand triazole bound, which produces conformer II only. Ferric cytochrome P450 was red in colour prior to the addition of the nitrogen bases and reduction by sodium dithionite. However, on reduction and addition of cyanopyridine the cytochrome P450, solution turned black, and on addition of triazole the solution turned a lighter shade of red when compared to the ferric cytochrome P450.

EXAFS analysis was carried out by Dr. Diakun, and showed that the Fe in ferric CYP2B4 at -70 °C was 5 coordinated, which is contrary to the findings of other authors, namely Dawson et al (1982) who suggested that the Fe existed in a 6 coordinated state. Analysis of the cyanopyridine and triazole bound cytochrome P450 revealed the Fe to be in a 5 coordinated state with a slight difference between the two samples. However, whether this difference was due to the presence of a thiol or thiolate ligand could not be determined. The unexpected result showing the ferric cytochrome P450 to be in a 5 coordinated state meant that results from this experiment could not be validated and suggests that low temperatures such as -70 °C may have affected the coordination state of the Fe.

4.4.9 Investigation into the role of the threonine residue.

Total protein and haem content.

From initial investigations it was found that the platelet homogenate contained 52 mg/ml of protein, with an actual haem content of 0.8 mg/ml. These values compared favourably with those reported by other authors (Haurand and Ullrich 1985), who reported a protein content of 50 mg/ml with a haem content of 0.9 mg/ml. All the following experiments were carried out with 4 mg/ml of protein.
in the sample cuvette.

**Binding of ligands to reduced thromboxane synthase.**

Microsomal samples of reduced thromboxane synthase in the presence of carbon monoxide have been reported to produce a spectrum consisting of two peaks with wavelengths ranging from 421-427 and 450-454 nm, some authors have suggested that the peak at 427 nm was due to the presence of 1-benzylimidazole, a well known thromboxane synthase inhibitor (Ullrich and Haurand 1983; Cinti and Feinstein 1976). Pure thromboxane synthase has been shown to produce only one peak at 450 nm when it was used as the enzyme source in the presence of carbon monoxide (Haurand and Ullrich 1985). It has not been established whether the two peaks on the binding of carbon monoxide to the reduced microsomal preparations of thromboxane synthase indicates the presence of denatured P420 as well as P450 or some other ligand possibly carbon monoxide in conformer II. If the peak is attributed to the presence of the second conformer, this is the first time it has been seen. The binding of carbon monoxide to reduced pure thromboxane synthase has been reported to be very slow, with final completion of the binding occurring after 30 minutes. The slow formation of the P450 peak was attributed to the presence of benzylimidazole which competes with carbon monoxide as a ligand for the enzyme (Ullrich and Haurand 1985; Cinti and Feinstein 1976).

The binding of carbon monoxide to the microsomal preparation of thromboxane synthase produced during the purification process outlined in section 4.3.9.1 was found to be instantaneous, producing a spectrum consisting of two peaks at 451 and 422 nm (table 4.10), whose absorbance and relative intensity did not change over 10 minutes. The binding of azines to the reduced enzyme produced spectra (table 4.10) similar to those reported for the binding of these bases to
CYP2B4 (table 4.1). The addition of pyridine at pH 6.0, produced a spectrum consisting initially of two peaks (422, 447) in a ratio of 2:1, with the major peak representing conformer II. The intensity of this peak increased with time at this pH with a concomitant decrease in the 447 nm peak (conformer I). The addition of carbon monoxide to the sample produced a shift in wavelength from 447 to 451 nm and from 422 to 420 nm, with the 420 peak as the predominant peak which is indicative of denaturation. The binding of pyridine to reduced thromboxane synthase at pH 7.0, produced a spectrum consisting of two peaks in equal proportions, however, with time the peak representing conformer II increased, indicating denaturation had taken place. The binding of the amines, hydroxylamine and trifluoroethylamine to thromboxane synthase produced spectra similar to those reported for CYP2B4 (table 4.3). The addition of hydroxylamine produced a spectrum consisting of one peak at 419 nm which increased in intensity with time. The addition of carbon monoxide produced a spectrum with a peak at 420 nm and no peak at 450 nm, indicating denaturation had occurred. The addition of trifluoroethylamine produced a spectrum consisting of two peaks of equal intensity, although identification of these two peaks was difficult. The addition of carbon monoxide to the sample produced a spectrum clearly showing two peaks at 453-454 nm and 419-420 nm, however the production of these peaks occurred against an initial spectrum consisting of two peaks. Since the initial spectrum of reduced thromboxane synthase consisted of two peaks no conclusive results can be ascertained from the binding of nitrogen bases to reduced thromboxane synthase.
4.4.10 Ligand binding to ferric CYP2B4.

The absolute spectrum of oxidised CYP2B4 has a Soret peak at 417 nm. The effect of binding different bases to oxidised CYP2B4 at different pHs was attempted to ascertain whether, as in the case of reduced P450 (table 4.1-3), pH has any effect on the degree of shift in wavelength observed on binding. Of all the bases used, pyridine, imidazole, N-acetylimidazole, methylamine, hydrazine and N-octylamine caused the greatest shift in wavelength all at a final concentration of 50 mM (table 4.11). The same shift in wavelength was observed for the aforementioned bases at all pHs used. The effect of time on the spectra obtained on the binding of bases to oxidised CYP2B4 was also investigated to determine whether any further slow reaction was taking place. It was found that after the initial binding of the nitrogen base, incubation at room temperature was found to have little effect on the spectral profile obtained.
### Table 4.11: The effect of pH on the binding of nitrogen bases to oxidised CYP2B4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peak value at pH (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.5</td>
</tr>
<tr>
<td>Pyridine</td>
<td>420</td>
</tr>
<tr>
<td>Pyridazine</td>
<td>417</td>
</tr>
<tr>
<td>Cyanopyridine</td>
<td>417</td>
</tr>
<tr>
<td>Imidazole</td>
<td>421</td>
</tr>
<tr>
<td>N-Acetyl imidazole</td>
<td>421</td>
</tr>
<tr>
<td>N-octylamine</td>
<td>422</td>
</tr>
<tr>
<td>Methylamine</td>
<td>420</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>420</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>417</td>
</tr>
<tr>
<td>Trifluoroethylamine</td>
<td>418</td>
</tr>
</tbody>
</table>

4.4.11 Determination of binding constants.

The binding constants for pyridine and N-acetylimidazole had previously been determined in section 3.4.2 and were found to be pH-independent (table 3.3; fig 3.5-6). The binding constants for these two compounds were determined in conjunction with identifying the sixth ligand in ferric CYP2B4. The binding constants for the binding of hydroxylamine and trifluoroethylamine to reduced CYP2B4 (table 4.7) were so different that it was decided to calculate the binding constant at the ferric level. It was found that at pH 7.0 that the binding constants for both these amines were very similar (table 4.12), however, the value obtained for hydroxylamine was much lower than that for the binding to the reduced state and indicates that the binding to oxidised CYP2B4 was more difficult than to the reduced enzyme.
Table 4.12 A table showing the apparent binding constants for the binding of nitrogen bases to oxidised CYP2B4.

ND not determined

<table>
<thead>
<tr>
<th>Compound / pH</th>
<th>Binding constant values (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.5</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>ND</td>
</tr>
<tr>
<td>Trifluoroethylamine</td>
<td>ND</td>
</tr>
</tbody>
</table>
4.5 DISCUSSION

Pyridine when bound to cytochrome P450 in the reduced state can act as a denaturing agent converting P450 to P420 (Imai and Sato 1967a), as well as a ligand to the metal. Therefore, studies on the coordination of ligands to cytochrome P450 were carried out against a background of denaturation. Furthermore, confusion arose during these studies where a given ligand exhibited similar Soret bands to cytochrome P420 on binding to cytochrome P450, i.e. conformer II formation. However, the two can be readily distinguished by the reaction with carbon monoxide.

The binding of the three families of nitrogen bases, namely the azines, azoles and amines to reduced CYP2B4 produced Soret bands within the range 419-429 nm or 442-447 nm (tables 4.1-3), which can be reasonably assigned to conformer II and I respectively. When the ligands expected to show the α-effect (pyridazine and hydroxylamine) are not taken into account, the better donors (high pKa) favour the formation of conformer II in all three families, but shown most clearly by the azines for both partially purified and pure CYP2B4. The binding of pyridine, imidazole and trifluoroethylamine to the cytochrome P450 isozymes CYP2E1 and CYP4A1, showed an identical pattern to that observed with CYP2B4, in that those bases with a high pKa favoured the formation of conformer II and those with a low pKa favoured formation of conformer I. While hydroxylamine produced conformer I as was the case with CYP2B4. Although most of the nitrogen bases produced one peak in the Soret region corresponding to either conformer, some of the ligands studied produced a mixture of the two conformers, namely pyridine, 4-methylpyridine (picoline) and N-octylamine (table 4.1-3). The binding of pyridine and to a lesser extent picoline showed a pH-dependence, with low pH (6.0) favouring formation of conformer II and high pH (8.0) favouring conformer I. Similar changes in the
difference spectrum could be obtained by changing the pH over the pH range 6.5 => 7.5 in either direction after both reduction and the addition of pyridine i.e the pH dependence is labile and reversible even after the coordination of pyridine.

It was found that the ratio between the conformers II and I produced on the binding of pyridine to reduced CYP2B4 at a fixed pyridine concentration increased with pH suggesting that one proton (H+) was involved which is similar to the results shown that the binding of ethylisocyanide involved one proton (Peisach and Mannering 1975). Whereas at fixed pH the ratio was independent of pyridine concentration. These results were in contrast to those reported for ethylisocyanide where at fixed pH the ratio of conformers varied with concentration of isocyanide (Dahl and Hodgson 1978a-b). The binding of pyridine to reduced CYP2B4 was found to be a fast reaction which is in contrast to reactions reported for CYP2E1 and Lysine mutated CYP2E1 with pyridine in which the reaction was reported to be very slow (ie complete formation of the spectrum occurred over a period of time) (Imai et al 1994).

In contrast to the binding of pyridine to reduced CYP2B4 the binding of N-octylamine was very different in that several slow reactions were observed at pH 7.0 and 8.0 (table 4.3). At pH 7.0 a mixture of conformers was observed, but this only lasted for the initial 40 seconds. With time the spectrum resembled that of conformer II with the peak at 426 nm. In contrast at pH 8.0, the initial spectrum resembled conformer II, but with time (10 minutes) a spectrum consisting of two peaks at 426 and 446 nm was observed. These results with N-octylamine suggest that the formation of the conformers was very sensitive to pH and that both conformers can be obtained at the same pH. It can be concluded from this that the presence of these conformers is very dependent on subtle conformational changes and/or binding of a second N-octylamine molecule.
in the active site.

It has been previously suggested that hydrophilic compounds can either form conformer II or conformer I on binding to reduced P450 (Imai and Sato 1967a). However the results obtained from these studies (tables 4.1-3) suggest that there is no obvious correlation between the ratio of conformers (AII/AII) and the hydrophobicity of the ligand. The fact that with both the amines which cannot π bond and the heterocycles (azines and azoles) high pKa favours conformer II and shows that the major factor involved must be the increase in σ donor power of the base represented by increase in pKa and/or lowering the pH (increase in H+), it also suggests that any metal to ligand π bonding probably plays a minor role (tables 4.4-6). However, π bonding may play an important role in the binding of triple bonded C ligands (e.g CO and CN). Carbon monoxide produces conformer I whilst cyanide produces a peak at 432 nm for the complex P450 Fe(II)-CN-, which is virtually the same as that of cyanomyoglobin (431 nm) (Miyake et al 1969), and suggests that it can be ascribed to conformer II.

Hydroxylamine and pyridazine are anomalous in their binding to reduced CYP2B4, they both favour the formation of conformer II instead of conformer I as would be expected from their pKa's (table 4.5-6). The spectrum of the oxygen derivative, (Fe(II)-O2) is also known to be anomalous, showing a Soret band at 419 nm which is close to that of myoglobin (418 nm), but very different from that of chloroperoxidase, which also possesses Cys in its anionic form (RS-) as the proximal ligand. A common denominator appears to exist between oxygen, pyridazine and hydroxylamine, which is most probably associated with the presence of a lone pair of electrons on the uncoordinated α atom. All these ligands show an unexpected stabilisation of conformer II, and both hydroxylamine and oxygen contain an oxygen atom as the α atom and
produce a Soret band at the same wavelength (419 nm). Coordination of the very hydrophilic hydroxylamine in the hydrophobic cavity of cytochrome P450 was unexpected and implicates strong hydrogen bonding to the invariant distal Thr/Ser residues. This in turn suggests that hydrogen bonding to the lone pair of electrons on the α atom may be involved in the stabilisation of conformer II by oxygen, hydroxylamine and pyridazine. The presence of the α effect and the relationship between pKa and the production of the conformers are both shown here for the first time. The parallel shown by hydroxylamine to oxygen with the stabilisation of conformer II, which is associated with the α effect (either electronic or polar/hydrogen bonding) suggests that hydroxylamine is an ideal model for the anomalous behaviour of oxygen.

The apparent binding constants for a number of nitrogen bases, namely, pyridine, pyridazine, trifluoroethylamine, and hydroxylamine with reduced CYP2B4 were determined by the fairly rapid titration of reduced microsomes with the nitrogen bases at either pH 6.0, 7.0 or 8.0, with the exception of pyridine (pH 6.5 and 8.0) (Table 4.7). The binding of all the bases appeared to be instantaneous and the curves obtained from duplicate experiments corresponded to the binding of one base per Fe. The calculated binding constant value (K) for pyridine at pH 6.5 of 560 M⁻¹ (i.e log K = 2.8, Kd = 1/K = 1.7 mM) is in full agreement with the value of Kd ~ 2.0 mM reported by Imai and Sato for pyridine, but considerably less than that for ethylisocyanide (Kd = 4.1 μM, K = 2.4 x 10⁵ M⁻¹) (Imai and Sato 1967a). The calculated binding constants for pyridazine, trifluoroethylamine and hydroxylamine were found to be pH independent which highlights anomalies between the different bases which produce conformer II only (hydroxylamine) and conformer I only (trifluoroethylamine) (table 4.7). These results compare with and contrast results shown by other authors on the binding of oxygen and carbon monoxide (Dolphin et al 1979) and isocyanides (Ichikawa and Yamano 1968). The binding
of oxygen and carbon monoxide and the ratios produced were found to be pH dependent while the binding (log K) of isocyanides was found to be pH independent. These results reflect anomalies observed on the binding of ligands to reduced P450. Although there was not a significant difference between the binding constants for the nitrogen bases belonging to the same families, the value for the very hydrophilic hydroxylamine (6000-8000 M$^{-1}$) is very high when compared to that obtained for the fairly hydrophobic trifluoroethylamine (22-40 M$^{-1}$) and is very anomalous and highly unexpected since the active site is very hydrophobic.

Elucidation of the nature of the proximal ligand, either thiol/thiolate present in conformer II and I was attempted using the technique of EXAFS. The different conformers were formed by the addition of ligands, triazole (conformer II only) and cyanopyridine (conformer I only) to reduced CYP2B4. EXAFS analysis of these samples as well as a ferric sample of CYP2B4, revealed that the nature of the iron in the conformer samples was 5 coordinated. However, the Cys ligand whether thiol or thiolate could not be conclusively determined. The apparent formation of the 5-coordinated ferric state indicates that temperature has an affect on the coordination state of the enzyme.

Reduced chloroperoxidase has the spectral properties of a thiolate-ligated monooxygenase, but its functional abilities are predominantly those of a peroxidase, such as those of histidine-ligated horseradish peroxidase (Hewson and Hager 1978). The binding of isocyanides to chloroperoxidase produces a single peak at 452 nm (Sono et al 1985), which contrasts to the double Soret spectrum observed with cytochrome P450 (Imai and Sato 1966a-b). The single peak produced on binding of isocyanides to chloroperoxidase can be assigned to conformer I and is due to the presence of Cys as the thiolate (RS$^{-}$). The production of this single peak suggests that the formation of conformer I in
CYP2B4 on the binding of nitrogen bases with a low pKa is due to the presence of the Cys ligand in its thiolate form.

As previously mentioned, of all the members of the three families of nitrogen bases studied, pyridine, pyridazine and 5-chloro-N-methylimidazole when bound to reduced CYP2B4 acted as a denaturing agents. However, this phenomenon has yet to date not been studied for binding of nitrogen bases to ferric CYP2B4. Our investigations suggested that the binding of different nitrogen bases to the ferric form did not cause any denaturation (table 4.11), even when the sample was incubated at room temperature for a period of time as indicated by the lack of a peak at 416 nm (P420 in the ferric state) (White and Coon 1982), and by the subsequent reduction with dithionite and reaction with carbon monoxide producing one peak at 450 nm. This indicates that the ferric form of the enzyme is more robust than the reduced form of the enzyme (i.e. denaturation occurs easier in the reduced state).

4.6 CONCLUSION.

The emerging picture suggests that RS− and RSH are required at different stages of the catalytic cycle and that this requirement is met by provision of the suitably poised equilibrium (1),

$$[RS^- \Rightarrow Fe^{(II)} \Leftrightarrow X]_I + H^+ \Leftrightarrow [RSH^+ \Rightarrow Fe^{(II)} \Leftrightarrow X]_II$$

which can be manipulated through the associated conformation change. It has already been suggested that this conformation change may play an important role in transfer of the second electron (Tsubaki et al 1989), and that a proton-relay system including the distal Thr/Ser is involved in this transfer (Gerber and Sligar 1994). The results in this chapter emphasise the general availability of a proton-dependent equilibrium between two conformers, and establishes that this is associated with a significant change in the electron density on the Fe(II) ion.
In addition, the results in this chapter further demonstrate the anomalies that can occur especially in the rates of reaction (cf pyridine and N-octylamine) and the pH independence observed for all the log K values. Two general trends are observed for the first time in this chapter in that conformer II is favoured by an increase in basicity (pKa) and that the α effect stabilises conformer II in contrast to the pka value. In respect of the α effect the results show that oxygen is not alone in its anomalous behaviour, with hydroxylamine providing a perfect model for oxygen and providing evidence for oxygen anomalous behaviour.
CHAPTER 5

General Discussion
5.1 GENERAL DISCUSSION.

There is considerable interest in the diversity of reactions catalysed by the cytochrome P450 family of haemoproteins, where the proximal ligand is the Cys thiolate anion (RS\(^{-}\)) which is in contrast to the His imidazole ring common to other haemoproteins such as haemoglobin, myoglobin, haem oxygenase and the peroxidases. One of the most interesting reactions of cytochrome P450 is the hydroxylation of alkanes and unactivated alkyl side chains which is characterised by the following reaction:

\[
\text{O}_2 + 2\text{H} + \text{RH} \Rightarrow \text{ROH} + \text{H}_2\text{O}
\]  
(1)

where 2H represents the two reducing equivalents supplied by NADPH via a specific cytochrome P450 reductase enzyme. This hydroxylation reaction involves the reduction of the enzyme (Fe\(^{3+}\) to Fe\(^{2+}\)), followed by coordination of oxygen (O\(_2\)) and reduction of the oxygen to a key hydroxylating intermediate. A number of questions concerning this reaction remain unanswered, especially:

1) the nature of the key intermediate,
2) the role of the reduced iron ion (Fe\(^{2+}\)).

As hydrogen peroxide can be produced from the reaction of oxygen and hydrogen, \((\text{O}_2 + 2\text{H} + 2\text{e} \Rightarrow \text{H}_2\text{O}_2)\) (2), and the fact that this hydrogen peroxide so produced can react with substrates (RH) to produce the hydroxylated product \((\text{H}_2\text{O}_2 + \text{RH} \Rightarrow \text{ROH} + \text{H}_2\text{O})\) (3), the general reaction (1) can in principle be studied as two segments and can therefore contribute to providing answers to the posed questions.

To investigate the hydroxylation reaction involving cytochrome P450, a substrate was required which possessed a finite solubility and could be used in a standard assay. All these criteria were exhibited by lauric acid. The hydroxylation of lauric acid using various oxygen donors and sources of cytochrome P450 was found to produce the hydroxymetabolites \(\omega\), \(\omega-1\) and \(\omega-2\) in varying ratios.
(table 3.8), which compares favourably with those reported by other authors (Cajacob *et al* 1988; Romano *et al* 1988; Ellin and Orrenius 1976). The hydroxylation of lauric acid by hydrogen peroxide using pure CYP2B4 produced the hydroxymetabolites ω-1 and ω-2 in the ratio 3:1, with no ω metabolite. This is the first example of a purified enzyme catalysing the hydroxylation of lauric acid with hydrogen peroxide.

The key hydroxylating intermediate of reaction (1) and (3) has never been detected and there is no agreement as to whether the key intermediate retains intact the O-O bond derived from molecular oxygen or hydrogen peroxide as coordinated $\text{H}_2\text{O}_2^*$ or $\text{HO}_2^-$, or whether it possesses only a single oxygen atom as the formally pentavalent iron-oxene or ferryl (FeO$^{3+}$) complex which is analogous to compound I of the peroxidases (Guengerich 1990; 1991). It has recently been shown that the reaction of iodosobenzene with CYP2B4 produces the elusive ferryl intermediate which is represented by the production of a new peak in the spectrum at approximately 390 nm (Blake and Coon 1989). The use of iodosobenzene to form the ferryl intermediate therefore provides a means of testing whether the key intermediate in the reactions involving hydrogen peroxide (bleaching and lauric acid hydroxylation) was a single oxygen unit (FeO$^{3+}$) or a peroxide ($\text{H}_2\text{O}_2$/HO$_2^-$). We have tested for a single versus two oxygen unit in both haem degradation and lauric acid hydroxylation by hydrogen peroxide.

A comparison of the rates of decrease in absorbance at 417 nm using 15 mM hydrogen peroxide, 0.1 mM iodosobenzene and 0.1 mM iodosobenzene followed by the addition of 15 mM hydrogen peroxide added after a period of 8 minutes, showed that the reactions with hydrogen peroxide produced a gradual but eventually complete bleaching of the chromophore (fig 3.7). This decrease in absorbance followed first order kinetics in the reaction of haem and hydrogen peroxide and was pH-independent, with no indication of any new band or
intermediate, in addition to the diminishing band of the starting Fe\textsuperscript{3+} complex at 417 nm. This was the first study of kinetics of haem degradation with pure CYP2B4 or apparently any cytochrome P450.

In contrast to this, the reaction with iodosobenzene rapidly produced a new spectrum, which showed a weak band at 385 nm (fig 3.9; 3.11) which remains relatively unchanged with time (fig 3.9). This is in agreement with the findings of Blake and Coon (1989). The inhibition of the bleaching of the chromophore by the prior reaction with iodosobenzene (fig 3.9) indicates that the pathway for bleaching in CYP2B4 does not involve the ferryl complex. The bleaching or inactivation of the cytochrome P450 enzyme showed parallels to the action of hydrogen peroxide on haem oxygenase (Wilks and Ortiz de Montellano 1993). This degradation was attributed to a two oxygen unit (Fe-OOH) and not the ferryl intermediate, also the formation of the ferryl intermediate was also found to inhibit haem degradation by hydrogen peroxide. The degradation of the haem in haem oxygenase and CYP2B4 by hydrogen peroxide involving a two oxygen unit appears to be a common denominator between the two enzymes which is irrespective of the proximal ligand (histidine in haem oxygenase and cysteine in CYP2B4). Comparison of the yields of hydroxylated products (table 3.8) from experiments involving CYP2B4 (1 \mu M), lauric acid (0.05 mM) at pH 7.5 which were allowed to react for 10 minutes with either hydrogen peroxide (15 mM), or iodosobenzene (0.1 mM) with lauric acid added first or added after 8 minutes (formation of ferryl intermediate) clearly revealed product formation from the reaction with hydrogen peroxide, but none from the reaction with iodosobenzene, even with lauric acid added after 10 minutes with a minimum ratio of > 200 for the yield of products with hydrogen peroxide compared to the reaction with iodosobenzene. These results indicate that the CYP2B4 catalysed hydroxylation of lauric acid by hydrogen peroxide does not involve the ferryl intermediate and neither iodosobenzene or the preformed ferryl intermediate.
complex are able to hydroxylate lauric acid at any significant rate. Vaz and co-workers have reported that a CYP2B4-catalysed reaction (4), where R is cyclohexyl and R-H is cyclohexane, was supported by hydrogen peroxide but not by iodosobenzene (Vaz et al 1991).

\[ \text{RCHO} + \text{H}_2\text{O}_2 \rightarrow (\text{R-H}) + \text{HCOOH} \quad (4) \]

They concluded that the "generally accepted pentavalent iron oxene is not the oxidant" and proposed an initial addition of some Fe\(^{3+}\)-coordinated peroxide to the aldehyde carbonyl group. It has also been concluded by Akhtar and co-workers (1994) that the products formed from certain reactions of steroids catalysed by cytochrome P450\(_{17\alpha}\) could only be explained by the reaction of some Fe\(^{3+}\) peroxide complex with a ketone carbonyl group.

Simultaneous monitoring of the changes in the CYP2B4 spectrum with hydrogen peroxide, and an increase in the formation of the hydroxylated products showed that the rate of inactivation of the enzyme (t\(_{1/2}\) = 10 minutes) is faster than the rate of chromophore bleaching (t\(_{1/2}\) = 12 minutes). The shorter t\(_{1/2}\) and the change in ratio of the products from approximately 0:1:1 at the start of the reaction to approximately 0:3:1 at the end (table 3.7; fig 3.14) indicates that hydrogen peroxide attacks one or more amino acids residues in the active site to cause some conformation change and then reacts with the porphyrin ring and that the product ratio is sensitive to conformational changes.

A key question remains as to whether the reaction involving \(O_2\) and \(2H\) proceeds through the same intermediate as hydrogen peroxide. The effect of changing the oxygen donor on the ratio of the hydroxylated products has been well studied in the case of the non-haem methane monooxygenase which, like cytochrome P450, can hydroxylate alkanes using either oxygen with NADH and a reductase or hydrogen peroxide alone (Froland et al 1992). All the evidence from these reactions indicates a common (but unidentified) hydroxylating
intermediate. The ratio of products (ω-1, ω-2) from hexane for example, changes from 0.7 with oxygen to 13.3 with hydrogen peroxide; this was ascribed to a conformational change at the reduced level in the former case. The hydroxylation of lauric acid using CYP2B4 was found to produce the metabolites (ω, ω-1) in a ratio of 1:2 (table 3.8), which compares with published ratios 1:4 and 1:8 (Cajacob et al 1988; Tanaka et al 1990). The relatively minor variations in product ratios observed with CYP2B4 (between oxygen and hydrogen peroxide as oxygen donors) clearly do not exclude, and if anything support, the formation of a common hydroxylating intermediate for both reactions (1) and (3), i.e with hydrogen peroxide and O₂ + 2H as the oxygen source.

The nature of the sixth ligand in the resting state of the cytochrome P450 enzyme is still under debate; it is thought to be a solvent molecule either in the form of a water molecule (Hilderbrant et al 1994) or a hydroxide ion (Banci et al 1994). In order to investigate the nature of this sixth ligand, pH-dependent binding studies were carried out utilising the bases pyridine and N-acetylimidazole and the anion CN⁻. Since the pK of HCN is 9.2, the linear increase in log K values observed on the binding of cyanide to partially purified samples of CYP2B4 (fig 3.3) with pH (below the pK), indicates that the observed equilibrium, where good isosbestic points were obtained (fig 3.1a-e), corresponds to the coordination of free CN⁻ with the corrected pH-independent values of the true log K given in table 3.2. Analysis of the changes in absorbance elicited by pyridine and N-acetylimidazole, with increasing concentration showed that additional equilibria intrude above 50 % conversion. It has recently been shown that that substrate free cytochrome P450cam can bind two molecules of pyridine, one binds to the metal (K ~ 10⁴ M⁻¹), whilst the other binds in the hydrophobic pocket (K ~ 10³ M⁻¹) (Banci et al 1994). It can be assumed that the deviations observed on the binding of pyridine and N-acetylimidazole to CYP2B4 reflect the binding of an additional molecule in the
hydrophobic pocket, subsequent to coordination of the first to the ferric iron ion (Fe$^{3+}$). These three ligand binding constants for pyridine, cyanide and N-acetylimidazole appear to the first reported for the Fe$^{3+}$ state of any mammalian cytochrome P450. The calculated binding constants for all three compounds were pH-independent, thus suggesting that the sixth ligand is a water molecule (FeOH$_2$).

When the pH-dependent conformational change is ignored, any equilibria involving the substitution of coordinated water by an anion or bases would both be expected to be pH-independent, as observed experimentally (table 3.2-3). It is however difficult to envisage such equilibria involving the substitution of a coordinated HO$^-$ could be pH-independent for both anions and bases, since this would require some special, presumably not merely coulombic interaction which could differentiate HO$^-$ from CN$^-$, thiolates and probably most of all other anions. Until there is definite evidence for such a mechanism, it seems reasonable to conclude that the low spin, 6-coordinate form of Fe$^{3+}$ cytochrome P450 with a Soret band at 417 nm possesses water as the distal ligand.

In contrast, all peroxidases only coordinate anions with the compulsory uptake of a proton according to the following equation, where P represents the protein, including the site which binds the proton, and (OH$_2$) indicates that the iron may be 6-coordinate with water as the ligand or 5-coordinate.

\[
P.Fe^3(OH_2) + X^- + H^+ \leftrightarrow +HP.Fe^3.X^- + H_2O
\]

The binding constant for butyl isocyanide by the Cys-ligated chloroperoxidase remains unchanged over the pH range 3 - 6 (Sono et al 1986). Whilst for all the anions studied, from strong acids (HI) to weak acids (HCN) a pH dependence of the apparent binding constant was shown, which is related to the pK and indicates coordination of the undissociated HX or the equivalent coordination of X$^-$ to the metal and binding of H$^+$ at some second site. The
compulsory binding of an anion with the uptake of a proton, and the related proton-coupled reduction of the Fe$^{3+}$ ion, by all peroxidases probably reflects the mechanism for "activating" hydrogen peroxide. In other words, this represents the conversion of the relatively unreactive H$_2$O$_2$ into the more reactive HO$_2^-$, even at physiological pH by the co-operative coordination of HO$_2^-$ to the metal and the binding of H$^+$ at a second site. The availability of protein-free models for the proton-coupled reduction of Fe$^{3+}$ porphyrins has clarified the role of coulombic interaction in such proton-coupled equilibria. Cytochrome P450 lacks the peroxidase mechanism for activating hydrogen peroxide to form a ferryl intermediate, which involves the H$^+$-coupled coordination of anions, in agreement with evidence against the role of FeO$_3^+$ in either haem degradation or lauric acid hydroxylation.

The results in chapter 3 indicate that in both haem degradation and lauric acid hydroxylation the key intermediate, which initiates attack on the organic substrate, is not the ferryl complex (FeO)$_3^+$ and therefore must be closely related to the reagent hydrogen peroxide, either as H$_2$O$_2$ (pK 11.2) or HO$_2^-$ coordinated to the ferric iron (Fe$^{3+}$). The experimental results (section 3.4.11) have shown that the hydroxylation of lauric acid was pH independent over the pH range 7.5-9.0, and that haem degradation was also pH independent (fig 3.8), with the rate of bleaching increasing with hydrogen peroxide concentration up to at least 0.6 M hydrogen peroxide (table 3.4). The reaction of hydrogen peroxide with lauric acid at pHs below 7.0 produced an increase in the rate of hydroxylation, but significantly no change in the ratio between the ω-1 and ω-2 products. This increase in the rate at pHs close to that of the pK of lauric acid (HLA) is probably due to an increase in the amount of lauric acid binding in the active site. However, an increase in hydrogen peroxide concentration only causes a small increase in the yield of hydroxylated lauric acid per unit time (table 3.9), presumably due to an increase in the rate of enzyme inactivation.
The simplest explanation of all these observations involves the reversible formation of coordinated hydrogen peroxide according to equation (5), where only the distal ligand is given.

The hydroxylation of lauric acid (denoted by RH) as in (6), which only differs from the mechanism proposed by White and Coworkers (1980) in that they proposed fission of the O-O bond to generate a free HO radical which attacked the substrate in a subsequent step while we propose that fission occurs only in step with the hydrogen atom transfer.

\[
\begin{align*}
\text{H}_2\text{O}_2 + [\text{H}_2\text{O} \Rightarrow \text{Fe}^3] & \Rightarrow \text{H}_2\text{O} + [\text{H}_2\text{O}_2 \Rightarrow \text{Fe}^3] \quad (5) \\
\text{RH} + [\text{HO-OH} \Rightarrow \text{Fe}^3] & \Rightarrow \text{R}^- + \text{H}_2\text{O} + [\text{HO}^- \Rightarrow \text{Fe}^4] \Rightarrow \text{ROH} + \text{H}_2\text{O} + [\text{Fe}^3] \Rightarrow \text{ROH} + [\text{H}_2\text{O} \Rightarrow \text{Fe}^3] \quad (6)
\end{align*}
\]

These results also highlight a common denominator in the ability of hydrogen peroxide to hydroxylate alkanes when associated with a sufficiently strong acid, whether the proton; for example \(\text{H}_3\text{O}_2^+\) in hydrogen fluoride, which can react well below 0° C (Olah et al 1977), or certain Lewis acid such as \(\text{Fe}^3+\) in cytochrome P450. The results with \(\text{H}_3\text{O}_2^+\) suggests that other oxygen donors, including iodosobenzene (PhIO) are activated by hydrogen abstraction from RH by a coordinated oxygen donor, loss of PhI and return of HO to the radical \(\text{R}^-\), without prior conversion to the ferryl intermediate (\(\text{FeO}^3+\)); this might explain the short lived burst of hydroxylation of lauric acid by iodosobenzene catalysed by whole microsomes (Gustafsson et al 1979). Alkanes may also be hydroxylated by oxo complexes as shown for a well-characterised complex of \(\text{RuO}^3+\) isoelectronic with \(\text{FeO}^3+\) (Che et al 1991), but this remains to be established for cytochrome P450. There has been a natural tendency to assume one common intermediate for all combinations of oxygen donors, substrates and isoenzymes of cytochrome P450; the situation may be more complex and more direct evidence is required.
The enzymatic cycle includes the binding of the substrate, reduction of the iron from Fe$^{3+}$ to Fe$^{2+}$, reaction with oxygen, uptake of a second electron and reaction with the substrate (eg hydroxylation). The nature of the key intermediate has previously been discussed, and now the possible role of the reduced iron in this cycle will be discussed along with the possibility that some nitrogen bases may be analogues for oxygen and could explain the anomalous behaviour of oxygen.

The binding of nitrogen bases belonging to the three different families (azines, azoles and amines) mostly produced in all three families a single Soret band at either 419-429 nm or 442-447 nm which can be reasonably assigned to conformer II and I, respectively. However, not all of the bases examined produce a single peak in the Soret region. As previously established for the isocyanides (Imai and Sato 1967a), which produced a mixture of the conformers, pyridine, pyrazine, N-octylamine and 4-methylpicoline all produced spectrum consisting of the two conformers (tables 4.1, 4.3). The ratio of the two conformers formed was affected by pH, with conformer II favoured by low pH and conformer I by high pH. The production of the two-peaked spectra was found to exist in a pH equilibrium which could be shifted in favour of one conformer by a change in pH (increase or decrease) (fig 4.5). The discovery of this reversible equilibrium supports the work previously proposed by other workers (Imai and Sato 1967a). The binding to reduced CYP2B4 of the nitrogen bases was found to be instantaneous and fast with the one exception of N-octylamine, which was very different. The binding of N-octylamine produced several slow reactions at pH 7.0 and 8.0 with the ratio of conformers formed being very sensitive to pH, and even both formed at the same pH (fig 4.4). These results suggest that the presence of the conformers is very dependent on a subtle conformational change and/or binding of a second N-octylamine in the active site.
The work carried out on CYP2B4 and these three families of bases found that when the compounds expected to show the \( \alpha \) effect were not taken into account, the better donors (i.e those with high pK) favoured the formation of conformer II in all three families. This was most clearly shown by the azines (tables 4.4-6). In contrast, those ligands with a low pK favoured the formation of conformer I. Previously it had been suggested that hydrophilic compounds can form either conformer I or conformer II (Imai and Sato 1967a+b), however, these results (tables 4.4-6) indicate that there is no obvious correlation between the ratio of absorbance (\( A_I/A_{II} \)) and the hydrophobicity of the ligand as initially suggested. The parallel between the amines and heterocycles re-emphasises that the major factor involved must be the \( \sigma \) donor power of the base (i.e bascity) and not \( \pi \) bonding since the amines cannot \( \pi \) bond, whilst the heterocycles can. However, \( \pi \) bonding may play an important role with C-ligands. The formation of a single band for the P450 Fe(III)-CN\(^{-}\) complex at 432 nm and a pK of 9.2 can assign cyanide to conformer II. The triple bonded C-ligands therefore provide another graded series of increasing donor (and decreasing acceptor) power from carbon monoxide (conformer I only) through isocyanides (I and II) to cyanide (II only). The C and N-ligands together provide a self-consistent pattern in which the equilibrium (1) can be systematically displaced to the right by protonation and/or increasing the electron density on the iron in four families (azines, azoles, amines, and triple-bonded C-ligands).

\[
[RS^- \Rightarrow Fe(II) \Leftarrow X]_I + H^+ \Leftrightarrow [RSH^+ \Rightarrow Fe(II) \Leftarrow X]_{II}
\]  

The binding of pyridazine and hydroxylamine was found to be anomalous, with both favouring the production of conformer II instead of conformer I as expected from their pKa as in the case of trifluoroethylamine and cyanopyridine. As previously stated (section 4.1) the binding of oxygen to reduced cytochrome P450 is also known to be anomalous, producing a peak at 419 nm which is
close to that of myoglobin (418), but very different from chloroperoxidase (which also contains Cys in its anionic form RS~), which produces a red-shifted peak at 430 nm (Sono et al 1985). The anomalous binding of these compounds can be attributed to the presence of a lone pair of electrons on the uncoordinated α atom (termed the α effect). However, all three ligands show the unexpected stabilisation of conformer II and both hydroxylamine and oxygen have an oxygen as the α atom and produce a spectrum at 419 nm. The similar results of stabilising conformer II observed with hydroxylamine make it an ideal model to study the anomalous effect of oxygen, from which it can be concluded that there is an activation mechanism for oxygen to stabilise conformer II.

It has been postulated that the proximal Cys ligand is either in the thiol (RSH) or thiolate (RS~) form (Kahl et al 1976), which can exist in an equilibrium (RSH ⇌ RS~ + H⁺). Recent work carried out using the iron porphyrin microperoxidase 8 (MP8) in the reduced state and sulphur ligands has shown the thiol/thiolate ligands to be readily accesible trans to the His residue (Norris and Pratt 1995 in press). Identification of which form of the cysteine residue is present in the two conformers of CYP2B4 was attempted using the technique of EXAFS. However, the technique did not produce any conclusive results, in that the native ferric enzyme and the reduced ligand bound enzyme produced EXAFS signals showing the enzyme to be in a 5-coordinated state. This was attributed to the low temperature (-70 °C) used in the experiment. Although further work is required it can be assumed that conformer II contains Cys in its thiol form and conformer I contains the thiolate form, which exist in an equilibrium (1). This equilibrium provides a means for explaining the associated conformational change and provides a mechanism for changes in electron density on the iron. Furthermore, this equilibrium supports the suggestion by Traylor and co-workers (1981) that the dissociation and loss of superoxide (O₂~) would be promoted by
the strong donor RS\(^-\), thus, there may therefore be a conflict of interest between the need for RS\(^-\) at one stage of the enzymic cycle and for a poorer donor at another (i.e. coordination of molecular oxygen).

The binding of oxygen to cytochrome P450 appears to be associated with hydrogen bonding to the distal Thr/Ser residues (Gerber and Sligar 1994). The coordination of the very hydrophilic hydroxylamine in the hydrophobic cavity of cytochrome P450 with a log K value higher than for the hydrophobic trifluoroethylamine (table 4.12) is unexpected and suggests strong hydrogen bonding to the distal Thr/Ser. This in turn suggests that hydrogen bonding to the lone pair of electrons on the \(\alpha\) atom may be involved in the stabilisation of conformer II by oxygen, hydroxylamine and pyridazine. In addition to the Thr/Ser residues present in the active site being implicated in the binding of oxygen, they may also be involved in a conformational change which may play an important role in the transfer of the second electron (Tsubaki \textit{et al} 1989) in conjunction with a proton relay system (Gerber and Sligar 1994). These two residues are in fact absent from those cytochrome P450s that handle endogenous hydroperoxides and which do not therefore involve reduction to Fe\(^{2+}\) and interaction with oxygen (Song \textit{et al} 1993). The use of one of these cytochrome P450s, namely thromboxane synthase to investigate the role of the Thr/Ser residues and anomalies involving the \(\alpha\) effect, however, was inconclusive due the production of two peaks in the reduced carbon monoxide spectrum (table 4.10).

In summary, the results in chapter 4 emphasise the general availability of a proton-dependent equilibrium between the two conformers and establish that this equilibrium is associated with a significant change in the electron density on the Fe(II) ion. Furthermore, hydrogen bonding to the distal Thr/Ser may play a key role in the coordination of oxygen as well as hydroxylamine and pyridazine.
The similar results observed for hydroxylamine and pyridazine show that oxygen is not alone in its anomalous behaviour and that hydroxylamine is an ideal model for oxygen and provides an automatic explanation for oxygen's anomalous behaviour (ie α effect). The emerging picture also suggests that RS⁻ and RSH, or another weak donor are required at different stages of the enzymic cycle; conformer II (uptake of oxygen) and conformer I (uptake of second electron), and that this requirement is met by the provision of the suitably poised equilibrium (1), which can be manipulated through the associated conformational change.

The results presented on the role of a peroxide complex in both lauric acid hydroxylation and haem degradation by CYP2B4 confirm and extend the results of Vaz et al (1991), Akhtar et al (1994) and Blake and Coon (1989) on cytochrome P450 catalysed reactions and provide a natural explanation for the apparently close relationship between general reactions (1) and (2) and bleaching of cytochrome P450. These results also demonstrate a common denominator in at least the initial step of haem degradation between cytochrome P450 and haem oxygenase and demonstrate further a common denominator in the ability of hydrogen peroxide to hydroxylate alkanes when associated with a sufficiently strong acid such as H₃O₂⁺ or certain Lewis acids such as Fe³⁺. By analogy, it could be expected that coordination to Fe³⁺ could activate donors such as iodosobenzene (PhlO) towards hydroxylation via analogous reactions involving hydrogen abstraction from the substrate (RH) by the coordinated oxygen atom, loss of Phl, transfer of hydroxide to the radical (R⁻), without prior conversion to the pentavalent ferryl complex. In the past there has been a tendency to assume a single intermediate for all combinations of oxygen donors, substrates and isoenzymes, however, the situation may be more complex and direct evidence for or against the nature of the key intermediate in other reactions is clearly needed.
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