6 Structure and Function of Cytochrome P450 Enzymes

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6.1 SUMMARY

The cytochrome P450 (P450) enzymes are hemoproteins that normally insert one atom of molecular oxygen into their substrates. In mammalian systems, this catalytic process requires the transfer of electrons from NADPH to the P450 enzyme by NADPH-cytochrome P450 reductase (CPR) and/or cytochrome b_5. Many alleles exist of both the P450 enzymes and CPR, some of which give rise to major differences in the observed catalytic activity. The human genome contains a family of 57P450 proteins, of which approximately 15 are responsible for oxidative and reductive metabolism of drugs and xenobiotics. The other members of the human P450 family are devoted to the synthesis or processing of steroids, fatty acids, eicosanoids, and vitamins or have functions that are not yet understood. To uniquely identify each of the P450 enzymes, a systematic nomenclature system has been developed on the basis of their degree of protein sequence identity. This system facilitates recognition of the analogies between enzymes of the same or different species.

The crystal structures of several human P450 enzymes have been determined. The structures of these normally membrane-bound mammalian proteins have the same general fold as those of the soluble bacterial P450 enzymes. The spectroscopic properties of all P450 enzymes are similar and can be used to monitor the binding of substrates

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to the enzymes. These structural and electronic analogies indicate that the same basic catalytic cycle is involved in substrate oxidation by all P450 enzymes. This consensus cycle involves the binding of molecular oxygen to the ferrous iron of the P450 enzyme followed by reductive cleavage of the dioxygen bond to give a highly reactive ferryl species that directly oxidizes the substrate.

6.2 INTRODUCTION

P450 enzymes are heme-containing monooxygenases that add one atom of molecular oxygen to their substrates while incorporating the other oxygen atom into a molecule of water [1,2]. The overall reaction catalyzed by P450 enzymes can be written as follows, where RH is a substrate and ROH is the product formed by addition of an oxygen atom to it:

$$RH + NAD(P)H + O_2 + H^+ \longrightarrow ROH + NAD(P)^+ + H_2O \qquad (6.1)$$

A simple example is the conversion of toluene ($C_6H_5CH_3$) to benzyl alcohol ($C_6H_5CH_2OH$). NADPH is the reducing cofactor for mammalian P450 enzymes, but NADH is the relevant cofactor for most bacterial enzymes. The P450 enzyme in this process is responsible for converting molecular oxygen into a reactive, iron-bound species that can transfer one oxygen atom to the substrate within the enzyme active site. The activation of molecular oxygen and the insertion of an oxygen atom into the substrate are mediated by the heme iron atom. The catalytic cycle, however, requires the participation of auxiliary proteins whose role is to bind NAD(P)H, to uncouple the two electrons provided by this cofactor, and to deliver them singly to the P450 heme group. The auxiliary protein for the mammalian drug-metabolizing P450 enzymes is CPR [3], but two distinct proteins, a flavin reductase (e.g., adrenodoxin reductase) and a ferredoxin (e.g., adrenodoxin), are required for some of the mitochondrial and most of the bacterial P450 enzymes [3].

Genetic analysis has identified thousands of P450 enzymes, and scores of such proteins have been investigated at the biochemical level. This chapter focuses on the human P450 enzymes that are most relevant from the point of view of drug metabolism. Of the 57P450 enzymes encoded in the human genome (Table 6.1), approximately 28 catalyze critical steps in the biosynthesis or removal of cholesterol, the sterol hormones, the bile acids, the eicosanoids, vitamins, and other physiologically important factors [4]. The P450 enzymes involved in these biosynthetic processes have narrow substrate specificities because evolution has tailored them to process specific endogenous substrates. The reactions catalyzed by such enzymes include removal of the 14α-methyl group of lanosterol in cholesterol biosynthesis [5], cleavage of the cholesterol side chain to generate the 17-keto sterols, progesterone and testosterone [6], and the 10-demethylation and aromatization of testosterone that produces estradiol [7] (Fig. 6.1). Relatively specific P450 enzymes are also involved in the degradation of fatty acids and elaboration of eicosanoids [8,9].

A second subset of approximately 16 human P450 enzymes are primarily involved in the oxidative, and occasionally reductive, metabolism of xenobiotics. This includes most drugs and the broad and variable range of compounds to which an individual is exposed. In most cases, the P450 system introduces an oxygen functionality such as

TABLE 6.1 The 57 Human Cytochrome P450 Enzymes

Drug Metabolism		Fatty Acids and Eicosanoids	Unknown (Orphan)		Sterols and Vitamins	
1A1	2D6	2J2	2A7	20A1	2R1	24A1
1A2	2E1	4A11	2S1	26C1	7A1	26A1
1B1	2F1	4B1	2U1	27C1	7B1	26B1
2A6	3A4	4F2	2W1		8B1	27A1
2A13	3A5	4F3	3A43		11A1	27B1
2B6	3A7	4F8	4A22		11B1	39A1
2C8		4F11	4F22		11B2	46A1
2C9		4F12	4V2		17A1	51A1
2C18		5A1	4X1		19A1	
2C19		8A1	4Z1		21A2	

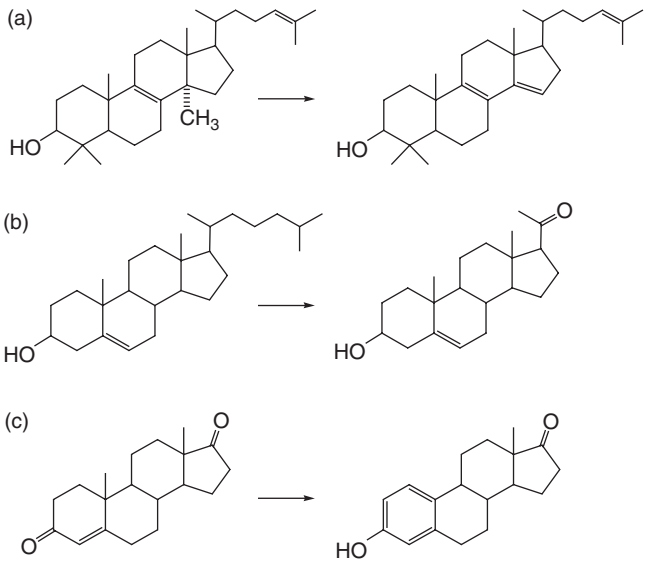


Figure 6.1 Biosynthetic transformations catalyzed by substrate-specific human P450 enzymes: (a) 14α-demethylation of lanosterol (CYP51) [5], (b) cholesterol side chain cleavage (CYP11A1) [6], and (c) aromatization of testosterone (CYP19A1) [7].

a hydroxyl or an epoxide into its substrates and can do so even at positions that are chemically difficult to oxidize. A given substrate may undergo multiple P450 oxidations catalyzed by one or several P450 enzymes. The functionalities thus introduced often serve as anchor points for the attachment (conjugation) of sulfate, glucuronide, or other polar moieties. The net result is the conversion of a lipophilic, poorly excreted molecule into a more polar product and finally, after conjugation, into a highly polar, ionic compound that is readily eliminated.

The P450 enzymes primarily devoted to xenobiotic metabolism have broad specificities, in accord with the fact that a small number of enzymes must cope with a

potentially very large and mutable diversity of chemical structures. Their broad specificity means that the enzymes can only exert limited control over both the types of substrates and the sites on those substrates that are oxidized. It is therefore not surprising that P450 enzymes, in the process of oxidizing compounds to more polar and excretable metabolites, sometimes convert a relatively innocuous molecule into chemically reactive and potentially toxic products. Examples of this are the epoxidation of bromobenzene, hydroxylation of phenacetin to give a highly reactive iminoquinone, and hydroxylation of the side chain of chloramphenicol resulting in the formation of an acyl chloride that can either be hydrolyzed by water to the acid or acylate proteins (Fig. 6.2) [10–12].

Some of the sterol hormone biosynthetic enzymes are concentrated in the adrenals, testes, and other steroidogenic tissues [4]. However, the xenobiotic-metabolizing P450 enzymes are not only present in most tissues but also found at particularly high levels in the liver, kidneys, lungs, gut, and nasal passages that are portals for the entry of xenobiotics into the body [4]. All the mammalian P450 enzymes are membrane bound and, with the exception of the seven steroid biosynthetic enzymes that are located in the mitochondria (Table 6.2) [13], are found in the endoplasmic reticulum of the cell. The mitochondrial enzymes are located in the inner membrane on the inner matrix side. The early biochemical work on the mammalian P450 enzymes involved solubilization and purification of the enzymes from native tissues, a historically important approach but one that was ultimately unable to fully resolve and establish the size and nature of the human P450 family. The study of these enzymes has been greatly facilitated by two advances: (a) sequencing of the human genome, which clearly identified the individual members of the family, and (b) the development of *Escherichia*

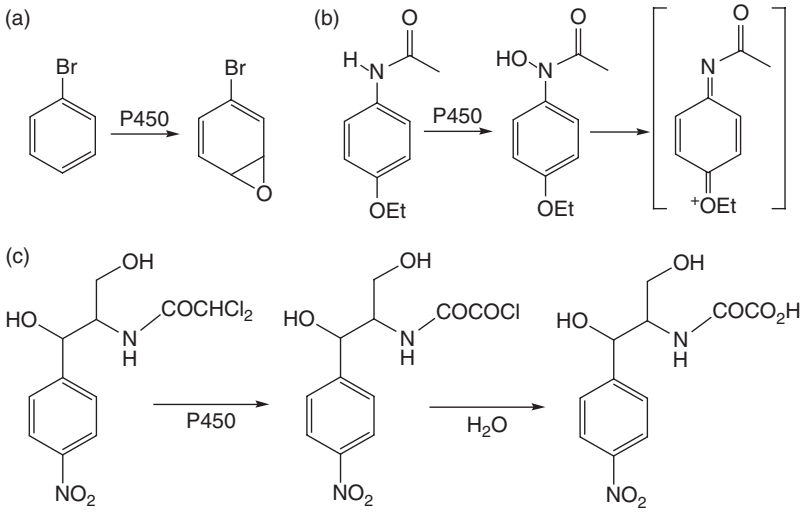


Figure 6.2 The oxidation of (a) bromobenzene to an epoxide derivative, (b) phenacetin to an iminoquinone that is responsible for the protein alkylation and toxicity associated with this drug, and (c) the oxidative dechlorination of chloramphenicol, which involves the formation of a reactive acyl chloride intermediate.

TABLE 6.2 The Mitochondrial Cytochrome P450 Enzymes and Their Location and Physiological Role

P450 Enzyme[a]	Function	Location
CYP11A (P450scc)	Cleavage of cholesterol side chain to give pregnenolone	Adrenal cortex, gonads
CYP11B1 (P45011β)	11β-Hydroxylation of 11-deoxycortisol to give cortisol	Adrenal cortex
CYP11B2 (P450aldo)	Aldosterone synthesis from 11-deoxycorticosterone	Adrenal cortex
CYP24A1	24-Hydroxylation of 25-hydroxyvitamin D_3	Kidneys
CYP27A1	27-Hydroxlation of cholesterol	Liver, kidneys
CYP27B1	1α-Hydroxylation of 25-hydroxyvitamin D_3	Kidneys
CYP27C1	Unknown	—

[a]The historical, nonsystematic name is given in parentheses.

coli, [14], *Saccharomyces cerevisiae* [15], and baculovirus/insect cell systems [16] for heterologous expression of the individual enzymes [17].

6.3 CYTOCHROME P450 NOMENCLATURE

The human genome encodes 57P450 enzymes (Table 6.1), several of which are considered "orphan" enzymes in that little is yet known about their substrate specificities or physiological roles. The initial nomenclature of P450 enzymes was based on their catalytic and physical properties, including the turnover of specific substrates, the relative migration of the proteins on SDS-PAGE, and their UV–vis absorption maxima. However, this initial nomenclature became unsatisfactory as the number of distinct P450 enzymes increased and related but nonidentical enzymes were found in different tissues and species. A systematic nomenclature was therefore formulated, in which P450 enzymes were classified into families and subfamilies based on the extent of their sequence identity [18]. The hypothesis underlying this approach is that enzymes that are more closely related in sequence, and therefore in evolutionary terms, are likely to exhibit a higher similarity in their catalytic and physiological functions. The basic principle in the new nomenclature is that two enzymes that share 40% or more amino acid sequence identity are grouped within the same family and two enzymes that share more than 55% identity are placed into the same subfamily. The sequence identity values that define the family and subfamily borders are somewhat arbitrary and, to some extent, can be adjusted if required by other evidence. In practical terms, P450 enzymes are assigned a number designating the family, a letter indicating the subfamily, and a second number that identifies the individual protein (Table 6.1). The names can be presented in two formats. One is a historically derived format in which the term P450 precedes the identifying numbers and the second a more systematic name in which the term CYP is similarly placed. Thus, both P4503A4 and CYP3A4 refer to the fourth enzyme of family 3, subfamily A. The name of the gene coding for the protein is the same as the CYP form, except that it is given in italics, that is, *CYP3A4*. In addition

to these systematic names, some of the enzymes that have been studied for many years still have trivial names based on the reactions that they catalyze. Three examples of this are lanosterol 14α-demethylase (CYP51A1), the cholesterol side chain cleavage enzyme P450scc (CYP11A1), and aromatase (CYP19A1). The terms CYP and P450 are often used as shorthand notations for cytochrome P450.

6.4 CYTOCHROME P450 ABUNDANCE, POLYMORPHISMS, AND VARIABILITY

There are 57P450 enzymes in humans, but the metabolism of drugs and xenobiotics by this enzyme system is primarily mediated by members of the CYP1, CYP2, and CYP3 families (Tables 6.1 and 6.3). The importance of a given enzyme in drug metabolism depends on its distribution within the body, its concentration in specific tissues, and the extent to which its substrate tolerance encompasses the xenobiotics to which it is exposed. In one study of human liver, CYP3A4 (including CYP3A5) accounted for ~30%; CYP1A2, ~13%; CYP2A6, ~4%; CYP2C9, ~20%; CYP2D6, ~2%; and CYP2E1, ~7% of the total P450 content [19]. An immunoquantitation study of human intestinal P450 enzymes similarly found that CYP3A4/CYP3A5 accounted for 82%; CYP2C9, 14%; CYP2C19, 2%; and other forms, 2% of the total [20]. *In vivo*, CYP3A4, the most abundant hepatic and intestinal P450 enzyme, and the closely related enzyme CYP3A5, which cannot be immunochemically distinguished from CYP3A4, account for more than 50% of all P450-dependent drug and xenobiotic metabolism. CYP2C9 and CYP2D6 are each responsible for approximately 15% of xenobiotic metabolism in the liver, and CYP2E1, CYP2C19, CYP2C8, CYP2B6, CYP2A6, CYP1B1, CYP1A2, and CYP1A1 contribute most of the rest [21,22]. However, as summarized below,

TABLE 6.3 Principal Human Cytochrome P450 Enzymes Involved in Drug Metabolism and Examples of Their Substrates[a]

Enzyme	Substrates
CYP1A1	*R*-warfarin, benzo[*a*]pyrene
CYP1A2	Acetaminophen, bufuralol, caffeine, clozapine, ethoxyresorufin, and theophyline
CYP2A6	Coumarin and nicotine
CYP2B6	7-Ethoxycoumarin and cyclophosphamide
CYP2C9	Benzphetamine, hexobarbital, phenytoin, tienilic acid, and tolbutamide
CYP2C19	Mephenytoin and omeprazole
CYP2D6	Bufuralol, debrisoquine, desipramine, dextromethorphan, propranolol, and sparteine
CYP2E1	Acetaminophen, aniline, caffeine, chlorzoxazone, and halothane
CYP3A4 and CYP3A5	Aldrin, Alfentanil, cortisol, cyclosporin A, dapsone, diltiazem, erythromycin, 17β-estradiol, ethinyl estradiol, lidocaine, nifedipine, quinidine, sterigmatocystin, taxol, testosterone, and warfarin

[a]This substrate list is drawn from the extensive review of Rendic and Di Carlo [23].

interindividual variations due to genetics, gender, diet, concurrent and prior drug exposure, and environmental factors can alter the relative contributions of the individual P450 enzymes.

The P450 enzymes, to a lesser or greater degree, are polymorphically distributed in the human population (Table 6.4) [4,21,22]. For example, owing to genetic differences, the CYP2D6 activity is relatively low in approximately 7–10% of Caucasians, a difference that decreases the ability of these individuals to oxidize debrisoquine and other compounds that are largely metabolized by this P450 enzyme [24]. Likewise, the concentration of CYP2C19 is relatively low in 20% of the Asian population and 4% of Caucasians, giving rise to an impaired ability to oxidize mephenytoin and other CYP2C19 substrates [25]. Many allelic variants of the human P450 enzymes (>80 for CYP3A4 alone) are now known, some of which do not significantly influence catalytic function but others greatly attenuate, completely suppress, or even increase the activity of a given enzyme. P450 polymorphisms have important consequences for drug metabolism and drug use [26,27].

The majority of drug-metabolizing P450 enzymes are *inducible*, a term that indicates that their concentration can be elevated by exposure to specific chemicals or environmental conditions. Enzyme induction can be the result of a receptor-dependent increase in expression or maturation of the active protein, posttranslational processing of the protein, or a decrease in degradation of the functional enzyme (Table 6.5) [29,30]. For example, CYP3A4 is induced by many drugs including the antitubercular drug rifampicin and the barbiturates, CYP2E1 by ethanol and isoniazid, and CYP1A1 and CYP1A2 by smoking, polycyclic aromatic hydrocarbons, and omeprazole. In animals, most persistent lipophilic substrates cause the induction of one or more P450 enzymes, and this is also true for the human enzymes. Enzyme induction is a temporal

TABLE 6.4 Examples of Some Cytochrome P450 Allelic Variants and Their Frequency in Caucasian and Asian Populations[a]

Enzyme	Allele	Activity	Allele Frequency	
			Caucasians	Asians
CYP2A6	*2	Inactive	3	0
	*4	Inactive	0.5	15.1
CYP2C9	*2	Decreased	10	0
	*3	Decreased	7	3
CYP2C19	*2	Inactive	13	25
	*3	Inactive	<1	8
CYP2D6	*2xN	Hyperactive	1.8	1
	*2	Decreased	32	—
	*3	Inactive	2	0
	*4	Inactive	21	1
	*5	Inactive	2	4.1
	*9	Decreased	2	—
CYP3A4	*2	Decreased	—	—
	*6	Decreased	—	—
CYP3A5	*7	Inactive	—	—

[a]The data in this table was taken from Ingelman-Sundeberg *et al*. [21], Brockmöller *et al*. [22], and Kee and Goldstein [28].

TABLE 6.5 Human Cytochrome P450 Enzymes and Typical Inducers

Enzyme	Inducer
CYP1A1	2-Acetylaminofluorene, omeprazole
CYP1A2	Phenytoin, omeprazole
CYP2A6	Phenobarbital, ethinyl estradiol
CYP2B6	Phenobarbital, phenytoin
CYP2C9	Phenobarbital, rifampicin
CYP2C19	Phenobarbital, rifampicin
CYP2D6	Not clearly demonstrated
CYP2E1	Ethanol, isoniazid
CYP3A4	Rifampicin, dexamethasone
CYP3A5	Rifampicin

For original literature references see Pelkonen *et al*. [29].

process, as it takes hours to days to fully induce a P450 enzyme and, once the inducing stimulus is removed, results in a gradual return of the enzyme to its resting level. The levels of individual drug-metabolizing P450 enzymes are thus influenced by recent prior exposure to drugs and xenobiotics.

6.5 SPECTROSCOPIC PROPERTIES OF CYTOCHROME P450 ENZYMES

The spectroscopic properties of the heme group, which are sensitive to the nature of the heme iron ligands; the oxidation state of the iron atom; and the polar and hydrogen-bonding nature of the environment around the heme provide important information on the P450 active site [31,32]. The P450 family of enzymes is distinguished from nearly all other hemoproteins by the UV–vis spectrum of their ferrous–CO complexes. While the intense Soret absorption maximum of this complex appears at ~420 nm for most hemoproteins, in P450 enzymes, it is split into two peaks with maxima at ~380 and ~450 nm. The peak at ~450 nm is specifically taken as the hallmark of the P450 enzymes and is the source of the name for this family of proteins. This unique absorption spectrum is due to coordination of a thiolate (RS^-) rather than a histidine or tyrosinate to the heme iron atom [32]. One of the first indications that a P450 enzyme is denatured is a shift of the 450-nm ferrous–CO spectrum to ~420 nm, as this shift signals that the proximal thiolate ligand has been protonated to a thiol ligand or has been entirely displaced from the iron by another functionality [33].

The ferrous–CO spectrum of P450 enzymes defines their spectroscopic signature, but the UV–vis spectra of the ferric enzymes are also informative. In the presence of a distal axial water ligand, the P450 iron atom is in the low spin state and the protein has a Soret absorption maximum at ~416–419 nm. Displacement of this distal water ligand, leaving the sixth iron coordination site vacant, gives rise to a high spin iron with a Soret absorption maximum at 390–416 nm. P450 enzymes can exist as an equilibrium mixture of the high and low spin states, but generally favor the low spin state. The exception to this is provided by enzymes such as CYP1A2 and CYP2D6 that do not appear to have a distal water ligand [34,35]. If the distal water ligand is present, its displacement by a substrate causes a shift in the absorption maximum from

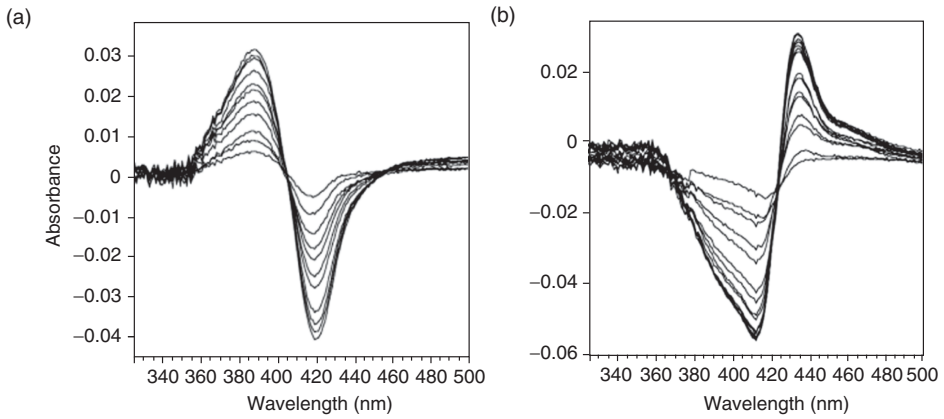


Figure 6.3 Typical difference spectra for the binding of ligands to a P450 enzyme. The spectra shown here are for the binding to bacterial CYP124 of (a) farnesyl pyrophosphate, giving a type I difference spectrum and (b) clotrimazole, giving a type II difference spectrum. In each panel, a spectrum was recorded after each incremental addition of the ligand to the sample cuvette. This figure, based on Ref. 36, was prepared by Dr. Jonathan Johnston.

~419 to 390 nm. This substrate-dependent spectroscopic shift is usually monitored by difference spectroscopy, a technique in which an exogenous ligand is only added to one of two otherwise identical enzyme-containing cuvettes (Fig. 6.3). The resulting difference spectrum, with a maximum at 385–390 nm and a trough at ~420 nm, is known as a *type I binding spectrum* [31]. A different spectroscopic shift is observed if the added ligand coordinates strongly to the heme iron atom, as this gives rise to a difference spectrum with a maximum at 425–435 nm and a trough at 390–405 nm. This so-called type II binding spectrum is observed, for example, when the nitrogen of an imidazole or triazole ring binds to the P450 heme iron atom. A type III difference spectrum with a maximum at 420 nm and a trough at 388–390 nm is obtained if the ligand only coordinates weakly to the heme iron atom. These spectroscopic changes are very useful, as they make it possible to spectroscopically monitor the binding of substrates and inhibitors to P450 enzymes. Exemptions to this approach occur if the resting enzyme does not have a distal water ligand or, occasionally, if the binding of a compound does not displace the distal water ligand.

6.6 STRUCTURES OF THE HUMAN CYTOCHROME P450 ENZYMES

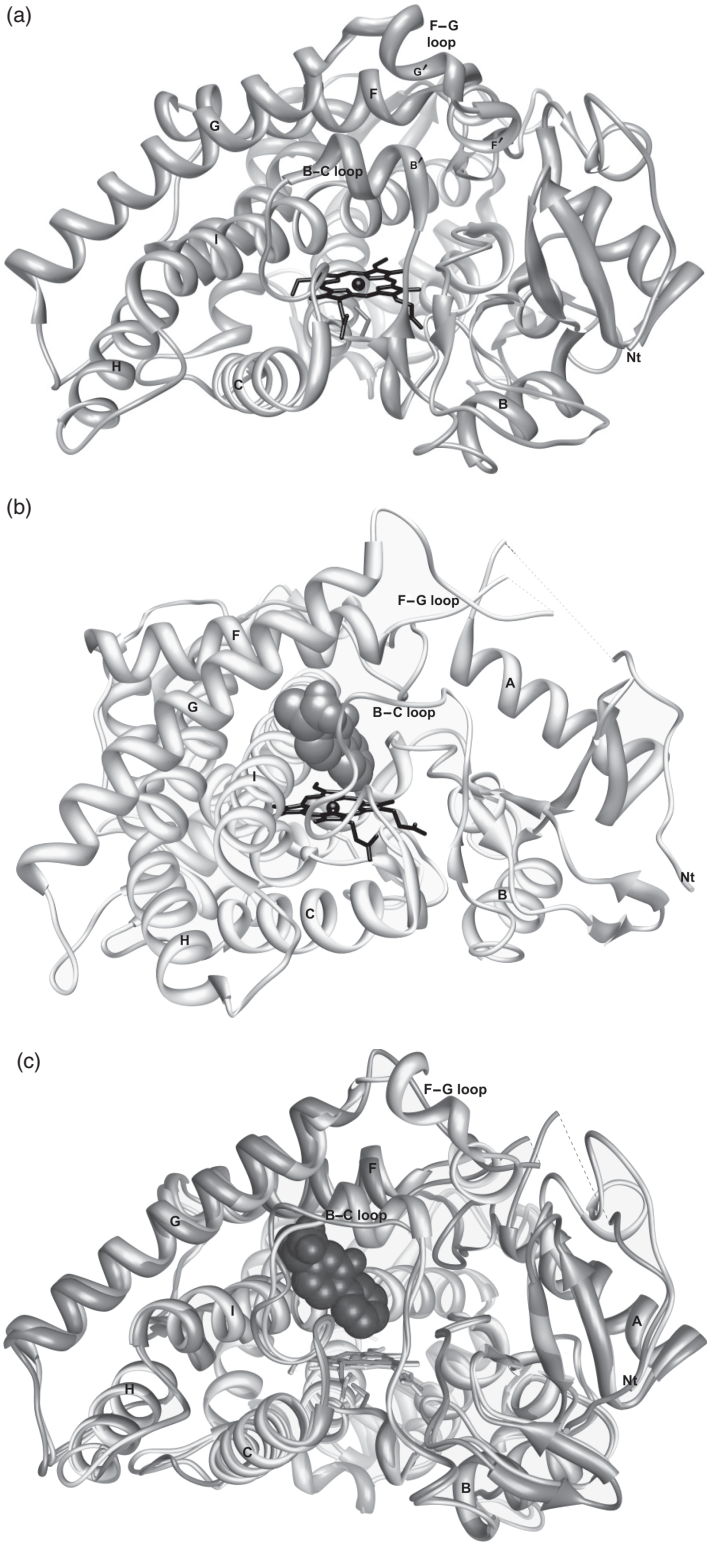
For many years, the only successful crystallizations and structure determinations of P450 enzymes were of the soluble bacterial enzymes. The structure of $P450_{cam}$ (CYP101), the first to be determined, was reported in 1985 [37]. The structure of $P450_{cam}$ and the subsequently determined structures of its complexes and other bacterial enzymes provided a wealth of information, much of which could be extrapolated to the membrane-bound mammalian P450 enzymes. However, 15 years passed before Johnson and coworkers [38] determined the structure of rabbit CYP2C5, the first of a mammalian, membrane-bound P450. The breakthrough represented by this structure has been followed at an increasing tempo by the determination of the

structures of human CYP1A2 [39], CYP2A6 [40], CYP2A13 [41], CYP2B4 [42], CYP2C8 [43], CYP2C9 [44,45], CYP2D6 [46], CYP2R1 [47], and CYP3A4 [48,49], often in the ligand-free state and in complexes with various ligands.

All the P450 enzymes have an approximately triangular prism shape that is dominated by 12 helices (Fig. 6.4) [38–49]. At the center of this structure is an iron protoporphyrin IX (heme) prosthetic group that is held in place by (i) the pincer action of the so-called I and J helices between which it is located, (ii) ion pairing and hydrogen bonds to the two carboxyl groups of the heme, and (iii) coordination of a cysteine thiolate to the proximal side of the heme iron atom. The catalytic action of P450 enzymes depends absolutely on coordination of this cysteine thiolate to the iron atom rather than a protonated cysteine thiol or some other ligand. As mentioned earlier, the ferrous–CO complex of the enzyme with a cysteine thiolate proximal ligand has a Soret maximum at ~450 nm, whereas this absorption maximum shifts to ~420 nm if the thiol is protonated [33]. The distal iron ligand, if one is present, is a water molecule. Given the complexity of the reactions catalyzed by P450 enzymes, there is surprisingly little in the way of catalytic machinery in the active site beyond the thiolate-coordinated heme group. The most important additional catalytic feature is a conserved threonine (or serine) residue on the distal side that (i) helps stabilize the ferrous–dioxy complex through hydrogen-bonding interactions and (ii) through similar interactions, promotes heterolytic cleavage of the dioxygen bond in the [Fe(III)–OOH] intermediate once it is formed. Replacement of this threonine by a non-hydrogen-bonding residue increases decoupling of substrate oxidation from oxygen utilization and results in a decrease or complete loss of substrate oxidation activity. This was first demonstrated for CYP101 ($P450_{cam}$) [50], but at approximately the same time was found with CYP1A2 to suppress oxidation of benzphetamine but not of 7-ethoxycoumarin [51]. Additional residues, exemplified by Asp251 in $P450_{cam}$ [52], appear to be important in maintaining a hydrogen-bonded network that helps deliver protons to the active site.

On the basis of the crystal structure of CYP101 and an alignment of this P450 sequence with those of the mammalian CYP2 family of proteins, Gotoh proposed that six regions of the mammalian proteins would be involved in substrate binding and recognition [53]. These substrate recognition sequences (SRSs) proved useful in the analysis of substrate binding to the human enzymes before structures for these enzymes became available. The rapidly expanding list of crystallographically determined human P450 structures now provides a more precise structural basis for the analysis of substrate binding and specificity. However, it is increasingly clear that P450 enzymes can undergo both small and large conformational adjustments to optimize the binding of their diverse substrates and inhibitors. Thus, the ligand in the structure of CYP2C9 with warfarin bound in its active site is located at a distance of more than 10 Å from the iron atom, whereas the structure with flurbiprofen in the active site places the substrate much closer to the heme iron atom (Fig. 6.4) [44,45]. The structure of flurbiprofen also

Figure 6.4 The crystal structure of human CYP2C9 in (a) the ligand-free state, (b) with flurbiprofen bound in the active site, and (c) superposition of the ligand-free and flurbiprofen-bound structures. In (c), dark corresponds to the flurbiprofen-bound structure and light color to the ligand-free structure. Flurbiprofen is in very dark tint with the heme below it in dark sticks. Some of the key structural elements of the protein are labeled. The conformational change that occurs on substrate binding is most notable in the F–G loop region. This figure was prepared by Dr. Sylvie Kandel. (See color insert.)


(a)
F–G loop
G′
F
G
F′
B–C loop
B′
I
H
C
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(b)
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F
A
G
B–C loop
I
Nt
B
C
H
(c)
F–G loop
F
G
B–C loop
I
A
Nt
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C
B

differs from that of the protein without a bound ligand, a difference most clearly seen in the F–G loop region of the protein (Fig. 6.4). The CYP2C9 protein in these two crystal structures is found to have undergone substantial conformational alterations. A second example is provided by the unligated structure of CYP2B4, which exhibits an open access channel into the active site [42]. A second structure with the protein complexed with 4-(4-chlorophenyl)imidazole shows the ligand in the active site close to the heme, with the access channel closed by the shift of a domain of the protein [54]. The dynamic plasticity of the P450 active site confounds efforts to understand and predict substrate specificity based on the limited set of human P450 crystal structures that are currently available.

6.7 ELECTRON-DONOR PARTNERS: CYTOCHROME P450 REDUCTASE AND CYTOCHROME b_5

Two electrons are required for each successful oxidation of a substrate by a P450 enzyme. In the case of the human drug-metabolizing forms of P450, these electrons are provided by CPR and/or cytochrome b_5 (Fig. 6.5) [55]. CPR is a 78-kDa protein with a hydrophobic N-terminal sequence that anchors it to the endoplasmic reticulum membrane. In contrast to the multiplicity of P450 enzymes, there is only one form of CPR. As shown by the crystal structure [56], the protein contains one FMN and one FAD that are used to uncouple the electrons provided by NADPH in order to deliver them one at a time to the P450 heme group. The enzyme is specific for NADPH and is not significantly reduced by NADH. The FAD group, reduced by hydride transfer from

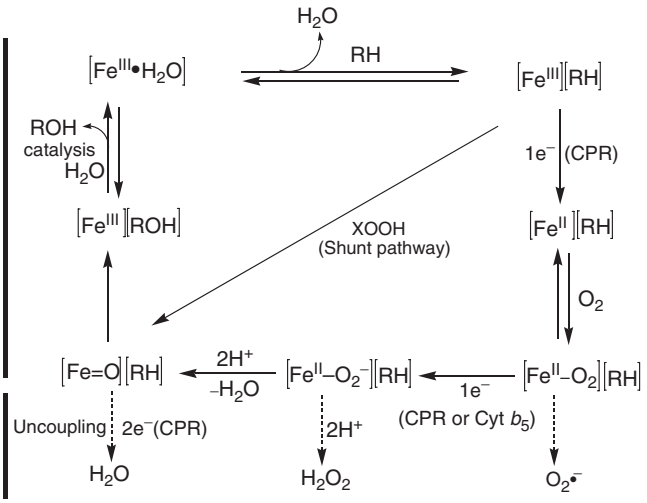

Figure 6.5 The catalytic cycle of most cytochrome P450 enzymes, including all of those involved in human drug metabolism. The iron in brackets represents the P450 heme iron atom; RH, a substrate; ROH, its oxidized product; and XOOH, a peroxide. The upper part of the cycle represents productive turnover in which the substrate RH is oxidized. In the lower part, turnover can also become uncoupled from substrate oxidation, producing superoxide, H_2O_2, and/or a second molecule of water. CPR, cytochrome P450 reductase; Cyt b_5, cytochrome b_5.

NADPH, transfers electrons to the P450 heme group via the FMN prosthetic group. With two flavin groups, CPR can accept up to four electrons, two for each flavin, but catalytic turnover usually involves cycling between the one- and three-electron reduced forms. Although truncated forms of CPR obtained by limited digestion of the endogenous membrane-bound enzyme or heterologous expression are able to reduce cytochrome *c*, they are no longer able to effectively reduce P450 enzymes. CPR, like the P450 enzymes themselves, is subject to polymorphic expression [57]. These polymorphisms are associated with disorders such as Antley–Bixler syndrome, the result of a perturbation of sterol hormone synthesis, but also have implications for drug metabolism [27]. Interestingly, CPR variants can interact differently with different P450 enzymes, so that the influence of a given CPR allele on one P450 enzyme cannot be extrapolated to another P450 enzyme [27].

With many P450 enzymes and substrates, electron transfer from CPR alone is sufficient to obtain maximum P450 catalytic turnover. However, with some enzymes, including CYP3A4, substrate oxidation can be enhanced or even modified by electron donation from cytochrome b_5 [58,59]. Cytochrome b_5, which can itself be reduced by either NADH/cytochrome b_5 reductase or CPR, is generally unable to deliver the first electron required to initiate P450 catalytic turnover [59]. However, it can deliver the second electron required to complete the catalytic cycle. One effect of cytochrome b_5 is therefore to enhance coupling of the electrons for the formation of the P450-activated species, an improvement that is achieved by decreasing the futile, uncoupled reduction of O_2 to H_2O_2 or water. Cytochrome b_5 may also influence P450 catalysis by allosteric mechanisms independent of its own electron-transfer ability, as illustrated by the finding that CYP3A4-catalyzed testosterone 6β-hydroxylation and nifedipine oxidation are stimulated by apocytochrome b_5 [58,60] and by the fact that cytochrome b_5 serves as a physiological control that helps determine which of the two possible products of sterol oxidation are formed by the sterol 17-hydroxylase/lyase (CYP17A1) [61].

6.8 CYTOCHROME P450 CATALYTIC CYCLE

The catalytic cycle of P450 appears to be the same regardless of whether the enzyme is from bacteria, plants, insects, or humans. Because the bacterial P450 enzymes are soluble and often more stable than their membrane-bound mammalian counterparts, much of the detailed work on the P450 catalytic cycle has been carried out with bacterial enzymes, particularly, CYP101 ($P450_{cam}$). However, a large body of the evidence obtained with the mammalian P450 enzymes confirms that the core mechanism of these enzymes is essentially invariant. As the high spin form of P450 enzymes is more easily reduced than the low spin form, catalytic turnover is initiated by the binding of a substrate, which displaces the distal water ligand if it is present (Fig. 6.5). In bacterial CYP101, the redox potential shifts from $E_{1/2} = -300$ to -170 mV upon binding of camphor, bringing the P450 within the range accessible to putidaredoxin, the electron-donor partner, which has a redox potential $E_{1/2} = -196$ mV [62]. Although a similar correlation of spin state with redox potential was initially not apparent for the mammalian enzymes reconstituted in artificial lipid mixtures [63], recent studies of CYP3A4 embedded in a more physiological nanodisc environment have given results consistent with those for CYP101 [64]. In these studies, substrate-free CYP3A4, which is 11%

high spin, was found to have an E° of -220 ± 10 mV, but the redox potential shifted on binding of erythromycin (22% high spin, $E^\circ = 210\pm10$ mV), testosterone (92% high spin, $E^\circ = -140\pm5$ mV), and bromocriptine (93% high spin, $E^\circ = -137\pm5$ mV). Electrons are transferred to these protein complexes by the FMN domain of one of the multiple oxidation states available to CPR [65].

Once the enzyme is reduced to the ferrous state by the first electron transfer, molecular oxygen binds to yield the ferrous–dioxy complex [Fe(II)–O_2]. This complex, unlike the analogous complex of myoglobin or hemoglobin, is not stable and, in the absence of further catalytic action, reverts to the ferric enzyme by the loss of superoxide. In the coupled catalytic cycle, however, the ferrous–dioxy complex is further reduced by a second electron from an electron-donor partner to the ferrous–peroxy complex [Fe(III)–OO–]. This intermediate is rapidly protonated to give the ferric hydroperoxide [Fe(III)–OOH]. The $P450_{cam}$ peroxy anion and hydroperoxide complexes have been observed spectroscopically at low temperatures [66]. Heterolytic cleavage of the dioxygen bond in the ferric hydroperoxide, which requires the uptake of a second proton and loss of a water molecule, produces a ferryl intermediate [1,2]. This ferryl intermediate is two oxidation states higher than the resting ferric state. It can formally be written as [Fe(V)=O], [Fe(IV)=O] with a porphyrin radical cation, or [Fe(IV)=O] with a protein radical, but the favored formulation appears to be [Fe(IV)=O] coupled to a porphyrin radical cation. This formulation is the same as that for the "Compound I" activated species of horseradish peroxidase, although the proximal iron ligand in that enzyme is a histidine nitrogen [67]. Efforts to detect the P450 "Compound I" ferryl intermediate, including cryogenic X-ray studies [66], have been tantalizing but ambiguous. However, recent investigations at the low temperature in which the reactive intermediate is generated by photoirradiation of a chemically generated [Fe(IV)=O] precursor provide strong support for its formulation as an [Fe(IV)=O]/porphyrin radical cation [68,69]. This ferryl intermediate is held to be the species that is directly responsible for most substrate oxidations.

Although the ferryl intermediate mediates most of the oxidation reactions, in a few P450 reactions, the ferric peroxy anion [Fe(III)–OO–] adds as a nucleophile to a carbonyl group, resulting in cleavage of the carbon–carbon bond between the carbonyl group and the parent structure [2]. A classic example is the 14α-demethylation of lanosterol, in which the enzyme (CYP51) oxidizes a methyl group first to an alcohol, then to an aldehyde, and in a final reaction involving the [Fe(III)–OO–] intermediate removes the carbon atom as a molecule of formic acid (Fig. 6.6). It has also been proposed that the ferric hydroperoxy intermediate [Fe(III)–OOH], in addition to serving as the precursor of the ferryl species, may itself oxidize some substrates. This intermediate, which is an electrophilic rather than nucleophilic oxidant, could conceivably oxidize thioethers, amines, olefins, and other easily oxidized functions. However, the evidence for a role of the [Fe(III)–OOH] intermediate in substrate oxidation remains unclear, as most of the evidence in favor of such reactions is derived from studies of mutants designed to impair the ability to form the ferryl intermediate (e.g., 70). This compromises the evidence because the role of the ferric hydroperoxide intermediate will depend on the relative rates of its conversion to the ferryl species versus its direct reaction with the substrate. Nevertheless, although its participation in hydrocarbon C–H bond oxidation is highly unlikely, it may yet prove to contribute in some situations to the oxidation of more easily oxidized functionalities.

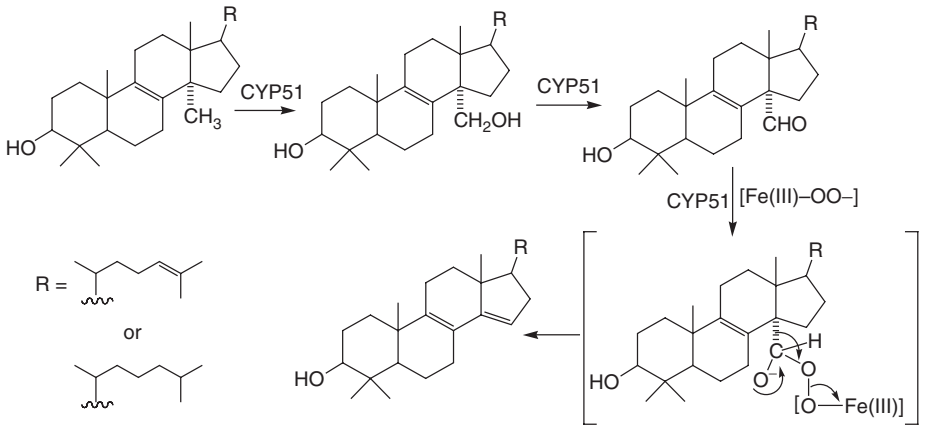

Figure 6.6 The three steps in the oxidative demethylation of lanosterol catalyzed by CYP51A1. [Fe(III)–OO–] represents the P450 ferrous peroxy anion intermediate.

An important parameter in P450 catalysis is the degree of uncoupling of oxygen consumption from substrate oxidation. As already indicated, the catalytic cycle traverses intermediates in which the heme iron atom is formally coordinated to a superoxide ion [Fe(II)–O_2 ↔ Fe(III)–$O_2^{\bullet-}$] or a hydrogen peroxide anion [Fe(III)–OOH]. Dissociation of these ligands can result in the formation of superoxide or H_2O_2, respectively, as the aborted products of P450 catalysis. Furthermore, there is evidence that the ferryl intermediate can itself be reduced by two electrons to a water molecule, regenerating the resting ferric enzyme. The importance of these uncoupling reactions depends on the specific P450 enzyme, the specific substrate, and the intervention or not of cytochrome b_5.

In vitro, many P450 enzymes can turn over their substrates with H_2O_2 rather than NADPH/CPR as the supporting system in what is termed the *shunt pathway*. In most cases, alkylhydroperoxides can be used instead of H_2O_2. In a sense, the mechanism of the P450 reaction with H_2O_2 is the reverse of autooxidation pathways, as the reaction is thought to generate the [Fe(III)–OOH] intermediate involved in normal catalytic turnover. The shortcoming of the "shunt" pathway is that peroxides readily damage the heme group and inactivate the enzyme, so that catalytic turnover can be fairly short-lived. It is unlikely that H_2O_2-dependent turnover of human P450 enzymes occurs to a significant extent *in vivo*, but the use of this approach *in vitro* has proven of value in investigating the mechanisms and specificities of the enzymes.

6.9 CONCLUSIONS

The P450 system is amazing in that by and large it can cope with the enormous variety of compounds to which mankind is exposed, particularly if one considers that only a dozen or so actual proteins are involved. A consequence of this metabolic efficiency is that highly reactive metabolites that are toxic or carcinogenic are not infrequently produced. The requirement for an efficient substrate oxidation system to enable the elimination of lipophilic xenobiotics thus carries with it the inherent potential to damage the host. This yin–yang balance is increasingly found in biology: one only

has to consider the cases of superoxide, nitric oxide, and carbon monoxide to find additional examples of biological two-edged swords.

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