7 Mechanisms of Cytochrome P450-Mediated Reactions

F. PETER GUENGERICH
Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN, USA

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7.1 INTRODUCTION

Most drugs are metabolized in the body, and understanding these processes is critical to the evaluation of pharmacokinetic suitability and their potential for toxicity and drug interactions. A generation ago, the lack of suitable human pharmacokinetic properties was the major cause of attrition of new chemical entities in the pharmaceutical industry [1]. Today, the fraction of failures due to poor pharmacokinetic properties is much lower (∼8% [1]), and the change can be attributed in large part to our improved understanding of the human enzymes involved in drug metabolism, including the transporters.

The fraction of drug metabolism reactions (i.e., for all drugs) attributed to P450s is ∼75% (Fig. 7.1) [2,3]. Of the 57 human P450 (*CYP*) genes, 5 account for 95% of drug metabolism [2,3]. In many cases, more than one P450 may be involved in the metabolism of a drug, even catalyzing the same reaction. The point should be made here that each P450 has the potential to catalyze many, if not all, of the types of reactions that discussed in this chapter because of the universality of the chemical mechanism.

The metabolism of a drug must be characterized in humans before it can be introduced into the market. The regulatory requirements have been made more stringent with the implementation of the Food and Drug Administration Guidance on Metabolites in Safety Testing in the United States [4], with similar International Conference on Harmonization guidelines now in place. Understanding metabolism, particularly, P450 reactions, is also important in considering and stratifying new chemical entities in pharmaceutical development, especially when there are issues regarding pharmacokinetics,

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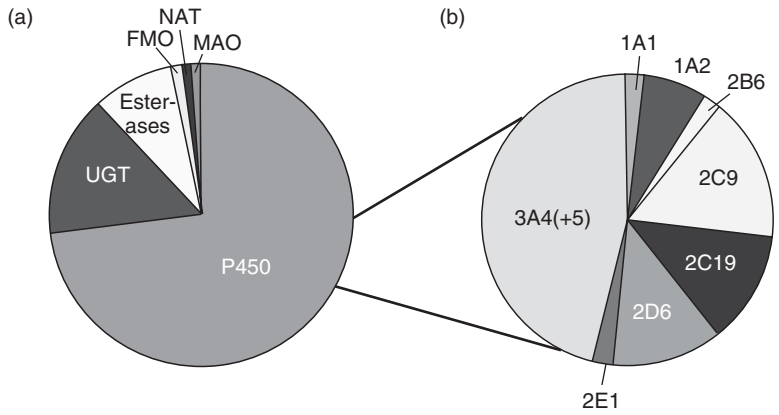

Figure 7.1 (a) Fractions of drug metabolism reactions catalyzed by each group of enzymes. (b) Fractions of P450 reactions catalyzed by the individual (human) P450s. UGT, uridine diphosphoglucuronosyl transferase; FMO, microsomal flavin-containing monooxygenase; NAT, N-acetyltransferase; MAO, monoamine oxidase. *Source*: Adapted from Williams *et al*. [2]. See also Wienkers and Heath [3].

drug interactions, and safety issues. Knowledge of P450 reactions may be critical in understanding the nature of glutathione (GSH) and macromolecular adducts and their implications.

7.2 OVERVIEW OF P450 REACTIONS

Before discussing the details of the P450 reactions, it is useful to consider the overall P450 cycle and which steps are most relevant to considerations in drug metabolism. A generally accepted scheme is shown in Fig. 7.2, although it must be emphasized that (i) the scheme shows only an electronic view of the reaction cycle (focused on the iron atom), without the changes in protein conformation that are known to be associated with various steps; (ii) some of the steps may have multiple steps within them (e.g., substrate binding); and (iii) this is not necessarily a linear cycle, for example, substrate may enter or come off at various states, not only at a single one.

The P450 cycle begins with the P450 iron in the ferric form. In step 1, the substrate binds. Such binding may or may not change the spin state or the oxidation–reduction potential. Step 2 involves the transfer of an electron from the flavoprotein NADPH-P450 reductase. This protein and the reaction have been studied extensively, and the system is not discussed here [5–7]. The ultimate source of the electrons is from NADPH, which transfers a hydride ion (two electrons) to the diflavin reductase, and the electrons flow through FAD to FMN to P450, necessarily one at a time. The rate of electron transfer to a P450 may or may not be enhanced by the presence of the substrate [8].

Step 3 involves the addition of O_2 to the ferrous iron. The resulting complex is unstable, and the rate of decomposition varies among P450s [9]. Step 4 involves the transfer of an electron to the ferrous–oxy complex ($Fe^{2+}O_2$). This can occur from NADPH-P450 reductase, although it has been much more difficult to study this reaction

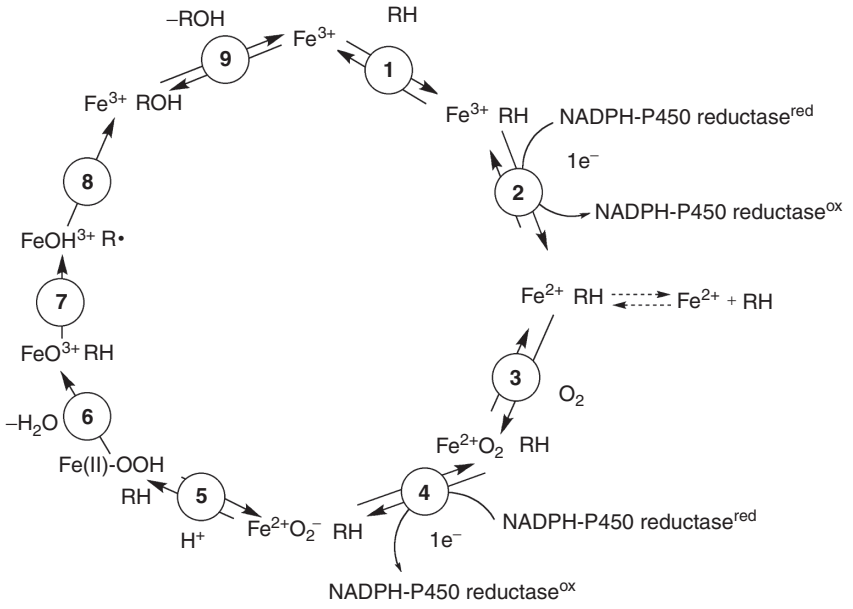

Figure 7.2 General catalytic cycle for P450 enzymes.

than step 2, so the nature of the electronic states is less secure. Cytochrome b_5 (b_5) can enhance some P450 reactions and has been shown to transfer an electron at this stage [10,11]. (Electron transfer from b_5 to P450 is possible at step 2—the difference in oxidation–reduction potential makes the rate slow [12].)

Following step 4, the reactions have been even harder to observe, and still much of our understanding of these steps is based largely on work with biomimetic models and, to some extent, theoretical considerations. The current understanding is summarized in Fig. 7.2. The FeO_2 complex can be depicted as $Fe^{2+}O_2^-$ and abstracts a proton; evidence exists that this is achieved via a Thr-based proton network in the I-helix [13,14]. As discussed later, there is speculation that the FeO^{2+} and $FeOOH^{3+}$ complexes can be the oxygenating species. However, (in the author's opinion) the current bulk of evidence supports the view that homolytic scission of the O–O bond to yield the FeO^{3+} complex (step 6) is a critical step in most P450 reactions. The FeO^{3+} complex has a strong precedent in peroxidase chemistry as "Compound I". However, attempts to characterize this intermediate in P450 have been difficult and controversy exists regarding exactly how stable it is [15–17]. In none of these reports has the intermediate been shown to form a product using unambiguous methods. Despite these experimental deficiencies, the FeO^{3+} complex is considered in all the mechanisms presented. The designation FeO^{3+} is used to avoid assigning electronic distribution to the iron atom, the oxygen, the porphyrin, and the Cys thiolate ligand (to the iron). The most common view of the electronic distribution is $Fe^{4+}O_2$-porphyrin radical$^{\cdot+}$.

Step 7 involves hydrogen abstraction (or one-electron oxidation) by the FeO^{3+} complex, yielding $FeOH^{3+}$ (Compound II, electronically equivalent to FeO_2^{2+}) plus a radical. In general, these radicals do not accumulate in P450 reactions as they do in peroxidase reactions. The rate of "oxygen rebound" (step 8) is considered fast

and estimated to be $\sim 10^{10}$ s^{-1} [18]. However, in some cases, rearrangements (of the substrate radical) are fast enough to occur and thus detect the steps.

The final step is release of the product to complete the reaction cycle. In general, most products seem to have affinities similar to the substrates.

7.3 P450 REACTIONS AND MECHANISMS

7.3.1 Substrate Binding

Substrate binding (step 1) is important in that the juxtaposition of the substrate in the P450 is critical to the reaction course, that is, the regio- and stereoselectivity, as well as the rate. Until recently, the nature of the P450 active sites was a matter of speculation or homology modeling based on a few bacterial P450s. Since 2003, structures have been obtained for the five human P450s most involved in drug metabolism—1A2, 2C9, 2C19, 2D6, and 3A4 [19–24] (2C19, Johnson EF, personal communication)—as well as for 2A6, 2A13, 2C8, 2E1, 2R1, 17A1, 19A1, 21A2, 46A1, and 51A1 [25–33]. The active sites vary considerably in size, from 190 to 1385 $Å^3$ (for 2E1 to 3A4, respectively) [23,27–29,34]. Just as importantly, the shapes of the active sites and various binding forces contributed by individual amino acids influence the binding of substrates. To elaborate on this last point, the size of the P450 2C8 active site is as large as that of P450 3A4 [23,34] but the shape is less "open," which may contribute to the narrower list of substrates. An example of how individual residues are involved is seen in P450 2D6 and the negatively charged amino acids Asp-301 and Glu-216 that interact with amine ligands [35–37]. The point should also be made that the shapes (and sizes) of the active sites are variable and change as the P450 binds different substrates, as judged from the crystal structures available to date. Thus, there is evidence that some aspect of induced fit occurs in substrate binding. However, the binding of ligands is clearly not so universal that every substrate can be bound by every P450.

One issue beyond the scope of this chapter is cooperativity. Cooperativity can be either homotropic or heterotropic. Homotropic cooperativity involves atypical interactions of the same substrate (or ligand), yielding positive or negative cooperativity for either binding or catalytic activity, for example, sigmoidal kinetics. Heterotropic cooperativity involves interaction of two different ligands and the ability of one to enhance the binding or catalytic activity of the other (*in vitro*, without any involvement of enzyme induction). These interactions are generally considered to involve two ligands within the active site of a P450, although few experiments have been done to eliminate the possibility of interactions due to binding at distant sites. In two cases, there is crystallographic evidence of multiple occupancy of a ligand in a human P450 [29,38], and some fluorescence evidence for direct interactions has also been reported [39].

Most P450s have the substrates imbedded in their interiors and must undergo conformational changes to open and close each time a ligand enters or exits. Some P450s can be described in a two-state system, either bound (with ligand) or unbound, and interchange is rapid. For instance, P450 2A6–coumarin binding can be characterized with a k_{on} of 2.7×10^6 M^{-1}/s (in the range of diffusion-limited reactions of proteins) and k_{off} of 5.7 s^{-1}. Thus, P450 2A6 must open and close for more than five times every second (at 23°C). Similar kinetic parameters were measured with the product 7-hydroxycoumarin [10].

Studies with P450 3A4 [40,41] and (rabbit) P450 1A2 [42] have provided evidence for a different course. The initial rate of interaction of ligands with these P450s is fast, as demonstrated by the rate of decrease in the fluorescence of the ligand α-naphthoflavone (αNF). However, the changes in the heme iron spin state are one to two orders of magnitude slower and follow a biexponential course [40]. Our interpretation is that the substrate binds rapidly at a site of the periphery (explaining the αNF quenching) and then moves slowly into the active site of the heme, in a position for catalysis (Fig. 7.3). The course of the events is slow enough to overlap the oxygenation process [40] and then complicate the kinetic analysis.

One explanation for biexponential kinetics of substrate binding is the existence of two or more distinct structural populations of unbound P450, for example, one in a boundlike structure (poised for binding) and a second structure ("ground state"), which are in slow equilibrium and bind substrate at different rates. In the experiments with the P450s 1A2 and 3A4 [40–42], results from pulsed mixing and other techniques argue against this explanation. However, the possibility of such phenomena cannot be dismissed for other P450 systems.

When P450s open and close to allow ligands to enter and leave, or (as in the cases of P450s 1A2 and 3A4) to migrate within the enzyme, particular residues along the "access" channels may be involved in the interactions. At this time, postulates of these mechanisms are only speculatory. Given the similarities of drug substrates and products of P450s (i.e., often the products are substrates for further oxidation), a strong case cannot be made for the existence of separate entrance and exit channels.

Finally, regarding practical significance of these issues, the point should be made that predicting metabolism is still difficult. Several bioinformatic strategies have been useful (e.g., MetaSite, Sporcalc, Glide, and Dock) [43], although most success has been achieved with systems based on prior experience with similar compounds as opposed to basic principles of chemistry and structure. Clearly it is more useful to have P450

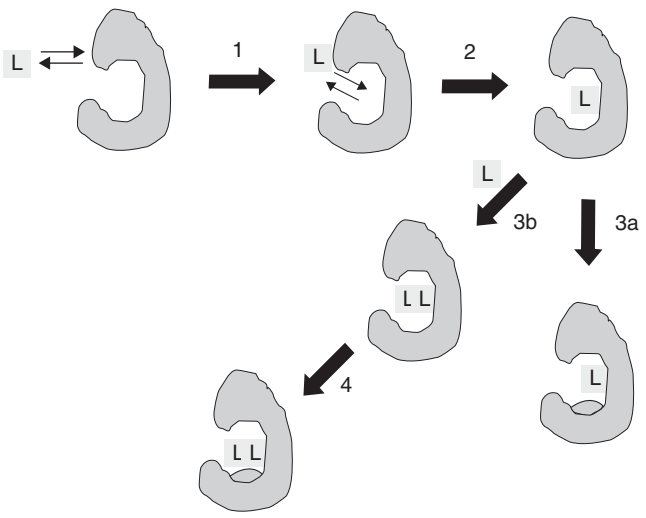

Figure 7.3 A proposed scheme for movement of a ligand into the active site of a P450, based on work with the P450s 1A2 and 3A4 [40–42]. L, ligand.

structures than not, but making predictions is still not trivial. As an example, the structure of the human P450 1A2–αNF complex [19] has the site of epoxidation positioned farthest from the iron [42]. Substrates can bind P450s in multiple ways, only some of which will be productive.

7.3.2 The Nature of the Active Oxygen

In early research on P450, the question of the nature of the active oxygenating species was a matter of interest. Much of the early discussion involved mobile elements such as superoxide anion ($O_2^{\cdot -}$) or some sort of an oxene species that could achieve the observed reactions. Such discussions were stimulated by evidence that NADPH-P450 reductase could generate $O_2^{\cdot -}$ [44]. While some findings supported this hypothesis [45], there were at least two major problems: (i) $O_2^{\cdot -}$ is nucleophilic but the nature of P450 reactions requires an electrophilic reagent and (ii) such a mechanism provides no control of regio- and stereoselectivity (or, in fact, even a role of the heme other than possibly reducing oxygen to a diffusible form).

Work in the area of biomimetic models suggested that a more appropriate model might be an electrophilic iron–oxygen complex [46], a view that developed further with metalloporphyrin models [47–49]. Another seminal study [50] showed that P450 reactions were associated with high intrinsic kinetic deuterium isotope effects and scrambling of stereochemistry, both of which could be interpreted as indicative of a reaction involving a radical intermediate. Further, typical P450 reactions occurred when P450 was mixed with single-oxygen donor "oxygen-surrogates" such as iodosylbenzene [51,52]. Thus, the view emerged that the P450 mechanism involves a course of events such as those shown in Fig. 7.4.

The P450–FeO^{3+} complex is electronically similar to peroxidase Compound I, which can catalyze some of the same reactions as P450s. The peroxidase Compound I is much more stable and has been extensively characterized, existing as an Fe(IV) (=O) complex with a one-electron deficiency in the porphyrin ring. The reduction of horseradish peroxidase Compound I occurs with $E_{m,7} \sim 1.0$ V, and the same $E_{m,7}$ has been estimated for the reduction of Compound II (of horseradish peroxidase) [54]. Similar values have been measured for biomimetic model metalloporphyrins [55,56]. A potential cannot be measured for the unstable P450–FeO^{3+} entity, but modeling and Marcus theory considerations suggest an even higher potential [57].

Although the perferryl FeO^{3+} species satisfies many criteria as the P450 oxygenating agent, alternate proposals have been developed and should be mentioned (Table 7.1). One unusual P450 reaction is the third step of the P450 19A1-catalyzed conversion of androgens to estrogens, involving the conversion of an exocyclic formyl group into formic acid and insertion of a double bond into a ring [58]. Model compounds have been shown to undergo similar deformylations [59–61]. One mechanism developed to explain this involves the $FeOO^-$ intermediate attack on the aldehyde [62]. Subsequent work with model P450 oxidations has led to the proposal that this reaction might be more general in P450 reactions [63]. Peracids are known to epoxide olefins, and even flavin 4a-hydroperoxides are now known to be involved in some epoxidations [64]. The possibility exists that some of the more facile P450 oxidations can be catalyzed by the $Fe^{2+}O_2(H)$ entity (Table 7.1). However, three points should be kept in mind: (i) theoretical considerations have been used to argue against a role of $Fe^{2+}O_2^-$ or $Fe^{2+}OOH$ in C-hydroxylation and epoxidation [65];

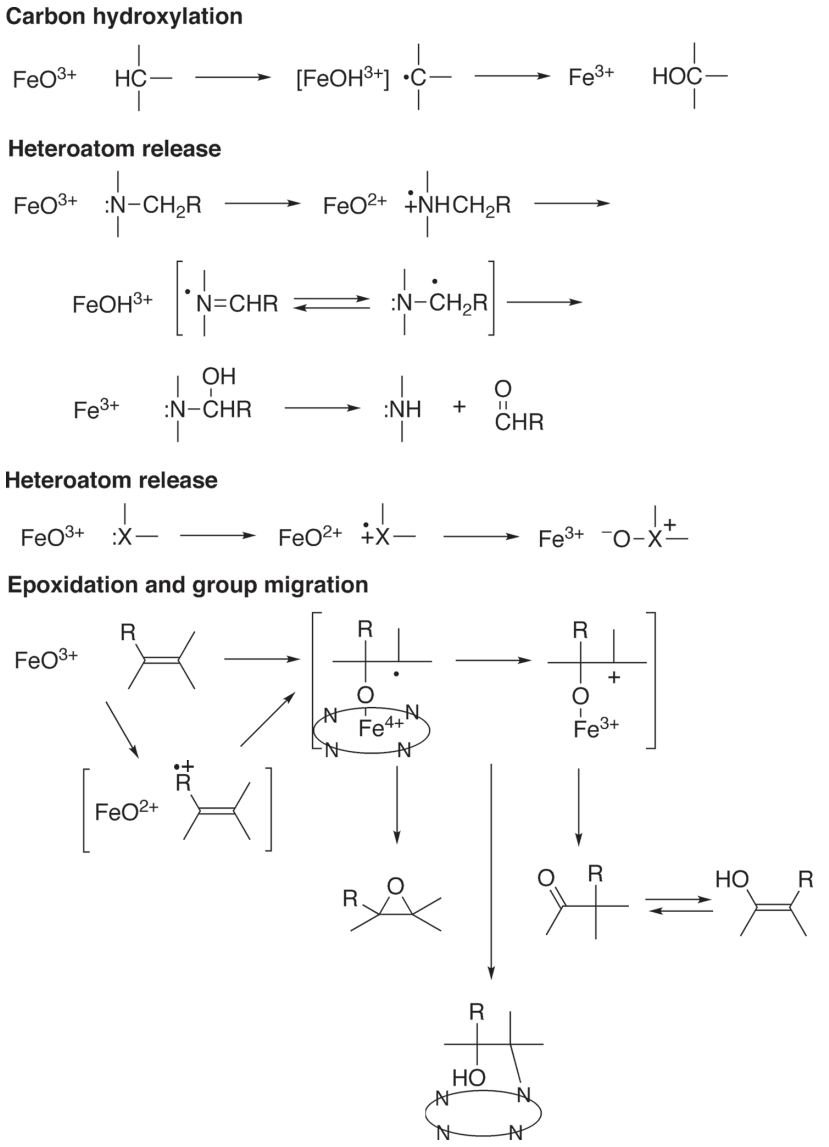

Figure 7.4 Generalized mechanism of P450 oxidation, based on FeO^{3+} chemistry [53].

(ii) many of the arguments for a role of $Fe^{2+}O_2$ in reactions rely on mutants in which the active site Thr was converted to Ala to disrupt protonation of the $Fe^{2+}O_2^-$ entity, but the interpretation of these active site mutants, as is often the case, may not be unambiguous; and (iii) even the aromatase deformylation step (see above) can be rationalized by a radical mechanism, which has some favor on theoretical grounds [66].

Alternate mechanisms have been proposed to explain apparent inconsistencies in "radical clock" experiments, that is, the lack of rearrangement of some molecules before the oxygen rebound [73]. The lack of rearrangement leads to very short estimates of

TABLE 7.1 Potential Iron–Oxygen Complexes Involved in P450–Catalyzed Oxidations[a]

Oxidation/Reaction Type	Some Potential Oxidations
FeO^{3+}/low spin (no barrier) [68]	C-Oxidation, epoxidation, X-oxidation
FeO^{3+}/high spin (no barrier) [68]	C-Oxidation, epoxidation, X-oxidation
FeO^{3+} (agostic) [69]	C-Oxidation
FeO^{3+} ($1e^-$ transfer) [70,71]	X-Oxidation
$FeOO^-$ [72]	Epoxidation
FeOOH [72]	Epoxidation, X-oxygenation

X, heteroatom.
[a]Ref. 67.

the lifetimes of intermediates (e.g., 10^{13} s^{-1} [74]), which are considered closer to transition-state lifetimes than intermediates. However, studies with other radical clocks have led to more consistent values for intermediate lifetimes [18]. The point should also be made that the strained cycloalkanes used as radical clocks may not necessarily undergo the same rates of ring opening inside the proteins (due to binding) as in solution and therefore lead to unusual values in some cases [75].

In part to explain some of the discrepancies in the radical clock results, Shaik has developed a two-state theory to explain alternate reactions that both proceed from a given substrate (Table 7.1) [68,76]. This is a theoretical system and has not been addressed in "wet" experiments. Details of the theory are beyond the scope of this chapter, but in short, the proposal has high and low spin configurations of the iron in the same FeO^{3+} entity, leading to different products. The proportions of high and low spin FeO^{3+} are a function of not only the P450 but also the substrate. Therefore, each system would have to be calculated (and the effort in calculation with a significant part of the protein is considerable in each case), so developing testable predictions is not trivial.

7.3.3 Considerations of Rate-Limiting Steps

In considering a particular P450 reaction, one question is which step (Fig. 7.2) is rate limiting. Associated with this is the even larger question of what really drives regioselectivity of reactions, beyond spatial and thermodynamic considerations. Some considerations are also in order regarding kinetic deuterium isotope effects, which may have new relevance in drug design [77].

In Fig. 7.2, step 1 has not historically been considered rate limiting, but the newer studies on the rates of movement into the active site with some P450s suggest that this aspect should be reevaluated in some cases [40]. Step 2, the reduction, has often been evaluated. It may be rate limiting in some cases [8], in that addition of extra reductase may stimulate some reactions [78,79]. Step 3 (O_2 binding) is not generally thought to be rate limiting, although in some cases P450s reduce substrates instead of oxidizing them, implying that the substrate competes effectively with O_2 for binding to ferrous iron.

Step 4 is often considered to be rate limiting because of the stimulatory effect of b_5. This appears not to be an artifact of the reconstituted system, in that anti-b_5 can also inhibit microsomal reactions [12]. However, electron transfer may not be the issue

[80–82]. If an electron is not transferred rapidly, the FeO_2^{2+} complex will dismute to ferric iron and $O_2^{\cdot -}$.

Little direct information is available on how rate-limiting step 5 is, in that this step cannot be measured. Site-directed mutagenesis can lower the rates of P450s, but these results do not provide an insight into rate limitation with wild-type P450s. Obviously, the complex will dismute if forward progress is attenuated. All of this discussion also applies to step 6.

Step 7 usually involves the breaking of a C–H bond and therefore is the only step amenable to interrogation by kinetic isotope experiments (except $^{18}O_2$ activation [83]). In numerous cases, kinetic deuterium isotope effects are seen and provide evidence that step 7 is the rate-limiting step, at least sometimes.

Step 8, oxygen rebound, has already been discussed. The rates are fast and do not limit the overall rate at which the P450 cycle (Fig. 7.2) operates, but the rate at which a radical intermediate rearranges will influence the distribution of products.

The final step (step 9) is product release. There are two ways of estimating this rate. One is to measure the apparent rate of product binding as a function of (product) concentration and extrapolate to zero concentration to obtain the off-rate. This experiment only works with a relatively low K_d. The other approach is to look for burst kinetics in a pre-steady-state kinetic experiment, with a burst indicating that the step following product formation is rate limiting [84]. Our own work has demonstrated the lack of rate limitation of step 9 in certain reactions catalyzed by the P450s 1A2 and 2A6 [10,85]. In the cases of oxidation of ethanol and acetaldehyde by P450 2E1, nearly quantitative bursts were observed [86,87]. However, considerations regarding the binding of acetaldehyde and acetic acid indicate that this slow step is probably a conformational change (undefined) instead of actual product release.

The point should be made that the concept of a rate-limiting step is too simplistic in a complex reaction cycle such as the P450 cycle (Fig. 7.2). The overall rate of product formation is limited not only by the forward rates of each step but also by the reverse rate constants, when reversible, and particularly by the rate of "side" pathways, for example, the loss of substrate at any of the several early points [10] and the formation of $O_2^{\cdot -}$, H_2O_2, and H_2O [10,88].

Some more discussion of kinetic isotope effects is in order, given the number of published studies and the confusion often attached to them. Most P450 reactions involving direct C–H bond breaking show appreciable intrinsic isotope effects, in the range of 3 to as high as 20 or more [85,89]. The intrinsic isotope effects are usually estimated using noncompetitive intramolecular experiments (with consideration of possible secondary isotope effects) or indirect approaches such as Northrop's method [90] or "branching analysis" [91]. Whether or not C–H bond breaking is rate limiting depends on the isotope effect measured in a noncompetitive intermolecular experiment, in which the (k_{cat}/K_m) parameter measured with a protiated substrate is divided by that measured with a deuterated substrate, with appropriate considerations (high fraction deuterium in deuterated substrate, no inhibitory impurities). This ratio is termed $^D(V/K)$, and the ratio of the two k_{cat} values is ^DV [90]. If these values approach the intrinsic isotope effect, then there is strong evidence for rate limitation. Obviously, if other steps are rate limiting then the isotope effect will not be expressed.

These considerations are important in light of recent interest in preparing deuterated versions of drugs to enhance pharmacokinetic properties [77]. Deuteration may or may not produce an isotope effect *in vivo*. Some drugs have produced *in vivo* isotope

effects [92]. One issue *in vivo* is which parameters to assess; the most useful are probably $C_{p,\,max}$ (maximum extrapolated plasma concentration) and clearance, which is related directly to the area under the curve (AUC). N-Dealkylations (of amines, not amides) generally show low kinetic isotope effects, because of their mechanisms, and the strategy would be less useful. To date, there has been no clear distinction as to which P450s show isotope effects, so phenotyping of the reaction if of little guidance.

7.3.4 Mechanisms of Oxidation of Major Reactions

The groupings of reactions will be slightly different than in previous reviews from this laboratory. With P450 oxidations covering thousands of different drug substrates and reactions, memorizing them all is impossible and, as in teaching organic chemistry, the only solution is to teach these in logical sets. Using the approach of teaching from chemical mechanism also provides a sound basis for the learner to deal with rather unusual reactions that seem too complex to discern at first observation.

Drug metabolism is best taught—in the author's experience—like organic synthesis, in that one starts with the product and then considers ways to work backward at each step, considering what is known about P450 (and other) reactions. One must appreciate nonenzymatic changes such as carbinolamine breakdown and ring formations. Starting to solve a complex biotransformation pathway by working forward is like designing a synthesis this way from crude materials—there are too many possibilities—so it should be worked backward. Of course, drug metabolism scientists may be called upon to predict possible transformations, but the likelihood of certain reactions happening is always difficult and experience is useful in this regard (particularly experience with similar compounds).

7.3.4.1 Carbon Oxidations (sp^3). The basic paradigm was discussed earlier (Figs. 7.5 and 7.6). The FeO^{3+} abstracts a hydrogen atom, and then oxygen rebound completes the reaction. A few variations on this theme follow.

In O-dealkylation of ethers (Fig. 7.5a), a similar hydroxylation occurs to yield a hemiacetal, which hydrolyzes. The same type of reaction can occur with esters, yielding an aldehyde and carboxylic acid [93] (Fig. 7.5b).

Another mode of the C–H cleavage is desaturation (Fig. 7.5c). This process is agreed to start with hydrogen atom abstraction. Instead of direct oxygen rebound, either a subsequent abstraction of the vicinal C–H atom or a nonenzymic rearrangement could occur to generate the vicinal radical, and the $FeOH^{3+}$ entity could abstract (H·) again at the original site. Either process would have the same stoichiometry and yield the desaturated product plus two molecules of H_2O. Desaturation reactions are usually accompanied by some alcohol formation, suggesting such a bifurcated pathway, but in at least one case desaturation appears to be extensive, without alcohol formation [94]. With ethyl carbomate, the subsequent epoxidation was much faster than desaturation and special methods were needed to quantify the olefin [95].

7.3.4.2 Heteroatom Oxidations (N, S, P, I). Most of this section applies to nitrogen, and the rest is applicable to sulfur and the other heteroatoms that often appear in drug heterocylces, and so on.

N-Dealkylations are considered to occur either by the hydrogen abstraction pathway described or, when the distance and oxidation potential are appropriate, by initial one-electron abstraction (Fig. 7.6). The aminium radical is unstable and, after α-proton

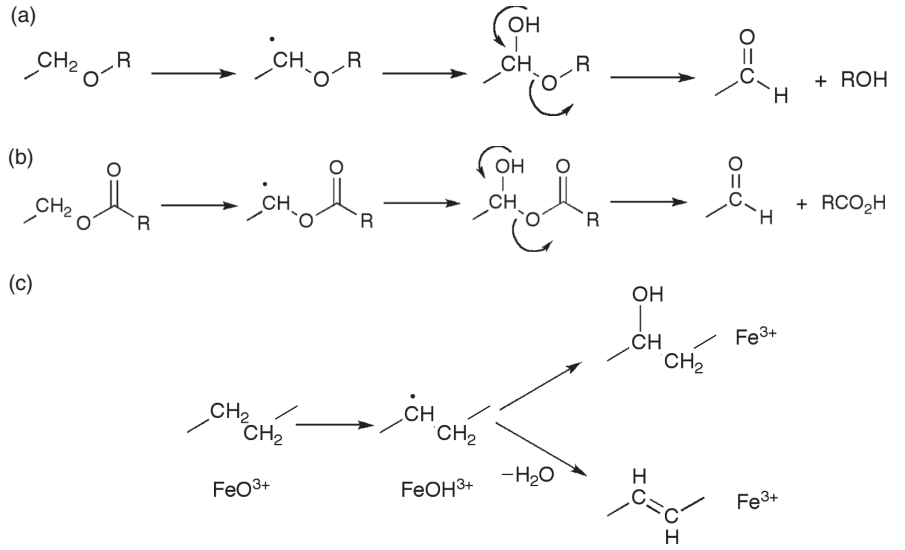

Figure 7.5 Carbon oxidation. (a) Oxidative cleavage of an ether, (b) oxidative cleavage of an ester, and (c) bifurcation between C-hydroxylation and desaturation reactions.

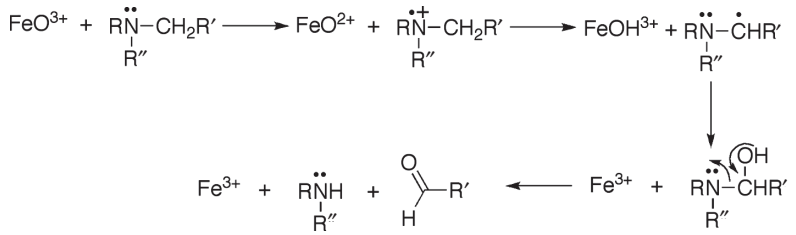

Figure 7.6 N-Dealkylation mechanism based on initial one-electron oxidation, and rearrangement of the resulting aminium radical.

abstraction, rearranges to a carbon radical for incipient oxygen addition, followed by breakdown of the carbinolamine. The FeO^{2+} is basic and helps abstract the α-proton, a mechanism not available in many peroxidases [96,97].

Several lines of evidence support the view that P450s N-dealkylate by the above pathway (although they are not excluded from hydrogen atom abstraction). (i) The low intrinsic kinetic deuterium isotope effects are consistent [97,98]. (ii) With dihydropyridines (which are vinylogous analogs), 4-alkyl groups are released and can be trapped as radicals (Fig. 7.7a) [99]. (iii) Other compounds with low oxidation potentials undergo characteristic rearrangements diagnostic for radical cations, for example, quadricyclane [100], or radical cations can be directly observed (e.g., 1,2,4,5-tetramethoxybenzene [101]). (iv) Rearrangements and mechanism-based inhibition with strained cycloalkylamines are observed and consistent with aminium radical formation [102–104]. (v) Analyses by Hammett and Marcus approaches are consistent with an aminium radical mechanism [57,105].

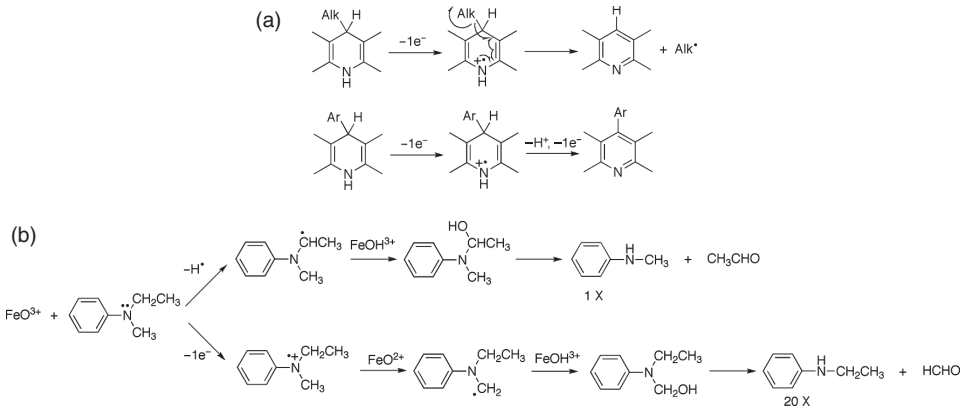

Figure 7.7 Two types of evidence in support of a one-electron transfer pathway (Fig. 7.6). (a) Oxidation of a 1,4-dihydropyridine with a 4-alkyl substituent leads to the extrusion of alkyl radical, which can be trapped, while oxidation of a 4-aryl analog yields to the retention of the 4-position moiety [99,106]. (b) Oxidation of *N*-ethyl-*N*-methylaniline by rat P450 2B1 yields a 20-fold excess of N-demethylation over the N-deethylation product [107].

Following abstraction of one electron, the resulting aminium radical rearranges to a carbon-centered radical. There is evidence that the FeO^{2+} (Compound II) entity acts as a base to facilitate this process [97]; most peroxidases only abstract electrons via the heme edge [96] and as a result are not capable of dissipating the aminium radicals and rebounding oxygen.

A similar process can be proposed for S- and P-dealkylations, in that the redox potentials are generally accessible. The point should be made that amides do not have low one-electron oxidation potentials, and therefore, N-dealkylation reactions of amides probably proceed largely via hydrogen abstraction. The higher kinetic deuterium isotope effects are consistent with this view [108].

Another point to be made is that the effective oxidation–reduction potential, $E_{(\mathrm{app})}$, is a function of the intrinsic potential, $E_{(\mathrm{int})}$, and the interatomic distance ($r_{1,2}$, in Å), as well as the dielectric constant (D).

$$E_{(\mathrm{app})} = E_{(\mathrm{int})} + \frac{14.4}{r_{1,2}D} \tag{7.1}$$

Thus, how far the nitrogen (or other heteroatom) is from the FeO^{3+} entity and the protein local dielectric constant influence the oxidation potential of the substrate.

Another experiment strongly implicating one-electron mechanisms is the N-dealkylation of *N*-ethyl-*N*-methylaniline by P450 2B1 [107], in which the ratio of N-demethylation to N-deethylation was 20:1, consistent with the higher stability of a methyl aminium radical but not with the inductive effect expected for methylene hydrogen atom abstraction [98] (Fig. 7.7b).

Oxygenation of heteroatoms is also observed with P450s. Two mechanisms can be considered. One is a one-electron oxidation, as discussed earlier (Fig. 7.8). This mechanism is intellectually satisfying in that it couples the different types of oxidations that occur with nitrogen and sulfur molecules and explains why oxygenation

Figure 7.8 Proposed N-oxygenation proceeding via one-electron oxidation.

is usually a very minor reaction when dealkylation is possible [105]. Even when N-dealkylation is favored, some N-oxygenation occurs, and the generally greater tendency of sulfur atoms to undergo oxygenation (rather than S-dealkylation) is consistent with greater radical stability. Interestingly, N-dealkylation of a series of substituted *N,N*-dimethylanilines yields a Hammett plot with a negative ρ value, consistent with stabilization of an aminium radical intermediate [105] but no such relationship was seen for N-oxygenation [107]. An alternate mechanism involves a direct transfer of oxygen to the heteroatom, without one-electron chemistry. Conceivably, this might even happen with $FeO_2(H)$, as proposed [109], instead of FeO^{3+} (Table 7.1). The point should be made that another microsomal monooxygenase, FMO, is prominent in the oxygenation of nitrogen and sulfur atoms [110], and care is needed to discern whether P450 or FMO is involved.

In heteroatom oxygenation, most of the focus is on nitrogen and sulfur, two atoms that often have low oxidation–reduction potentials. In general, the high oxidation potential of oxygen makes an electron-transfer pathway less likely, and most of these reactions proceed by the carbon hydroxylation pathway described earlier. Phosphorus oxygenation is known [111] and very feasible due to the low oxidation potential. Other heteroatom oxygenations involve halogens, as first proposed for dihaloethanes [112]. An iodosylbenzene-dependent oxygenation of iodobenzene was demonstrated by Burka *et al*. [113]. NADPH-dependent P450 oxygenation of aryl iodides and bromides was demonstrated later [114] using model compounds that trapped the resulting haloso products to yield stable entities. Although chlorine is harder to oxidize, He *et al*. [115] demonstrated that ω-chloro and ω-bromo fatty acids are oxygenated (on the halogen) by rat P450 4A1 (The products are unstable, and the analysis was based on the detection of isotopically labeled (^{18}O) alcohols, with the oxygen being derived from H_2O and not O_2.)

7.3.4.3 Reaction of π-Bond Substrates. This class includes mainly olefins, acetylenes, and aryl compounds. The bulk of the evidence is consistent with a stepwise mechanism in which FeO^{3+} reacts with a double bond to generate an intermediate, with a radical localized at the carbon vicinal to the C–O bond formed (Fig. 7.9). An epoxide can be formed by a hemolytic mechanism. Alternatively, electron transfer can generate a carbocationic intermediate. This intermediate could also close to form an epoxide, in a heterolytic process, or undergo a 1,2-shift of an anion (e.g., halide or hydride ion [116]) to generate a rearranged carbonyl product. In the case of aryl compounds, the aryl ketone rearranges to generate a phenol. This process is a part of the so-called NIH Shift (of hydrogen or other atoms) [117]. The point should be made that the demonstration of an NIH shift in the formation of a phenol is not definite evidence for the existence of an epoxide intermediate in the process; the mechanism may occur without the epoxide (Fig. 7.10).

Similar mechanisms can be written for the oxidation of acetylenes, with the collapse of an intermediate via a heterolytic path to form a putative epoxide [118], hydrogen

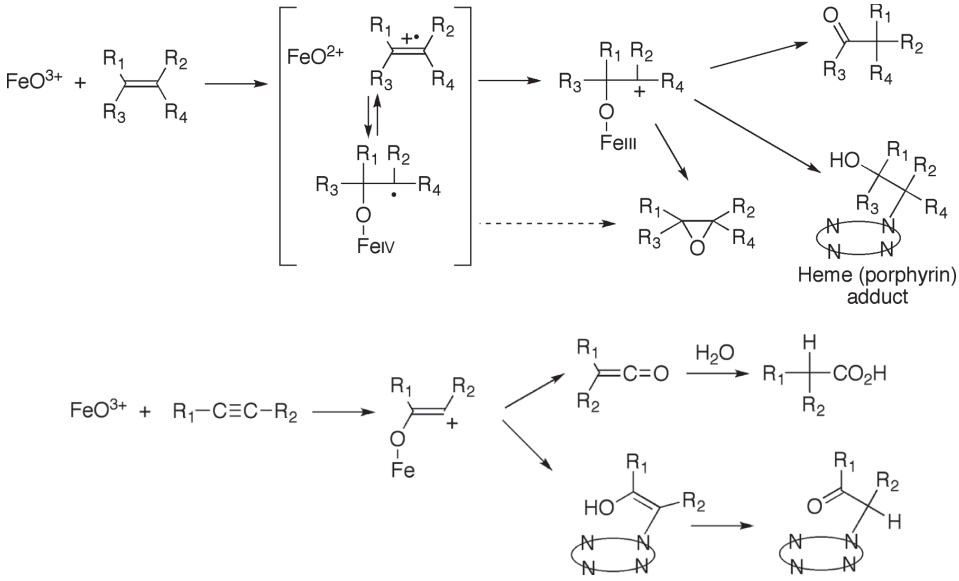

Figure 7.9 Some possible mechanisms of oxidation of olefins and acetylene.

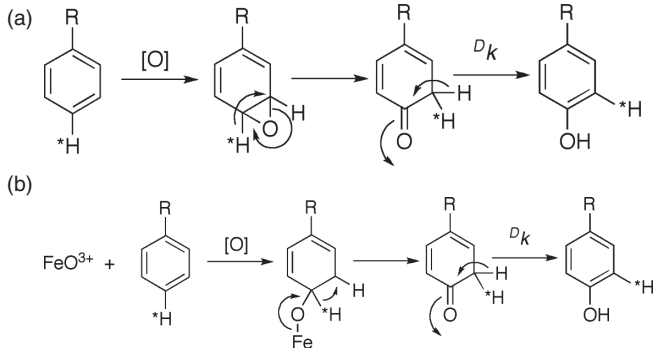

Figure 7.10 Aromatic hydroxylation and an NIH shift of a *para*-hydrogen [117] via two mechanisms: (a) concerted epoxidation followed by rearrangement and (b) stepwise electrophilic attack followed by rearrangement.

migration to generate a (reactive) ketene, or direct reaction with the heme porphyrin to generate an adduct (Fig. 7.9).

The view that a common mechanism drives these reactions is consistent with a number of lines of evidence and supported by kinetic hydrogen isotope effect results [119]. Thus, the mechanism can be considered to be nonconcerted. Another phenomenon that has been observed with some P450s and some of their substrates is proton exchange with water during epoxidation, which can only be explained with an intermediate involving a Fe–C bond [120]. However, an alternate point of view is that dual mechanisms are involved in the two-state system of Shaik [68]: a concerted low spin mechanism leads to epoxidation and a high spin nonconcerted reaction is involved in the formation of the other products [121].

7.3.5 More Complex Transformations of Drugs

Almost all P450 oxidation reactions can be explained with the basic mechanisms presented here, utilizing the perferryl FeO^{3+} entity and possibly $FeO_2(H)$, as already mentioned (Table 7.1). Some examples of seemingly more complex reactions have been presented elsewhere and are not recapitulated in this chapter [67,122], including ring expansion [104], ring contraction [123], and ring formation [124] (Fig. 7.11).

C–C and C–O bond coupling reactions are not unusual in P450 reactions involved in biosynthetic reactions in plants [126]. Such coupling reactions have now been observed with the (human) P450s 2D6 and 3A4 in the biosynthesis of mammalian alkaloids [127] (Fig. 7.12), and there are also some examples in drug transformations [128].

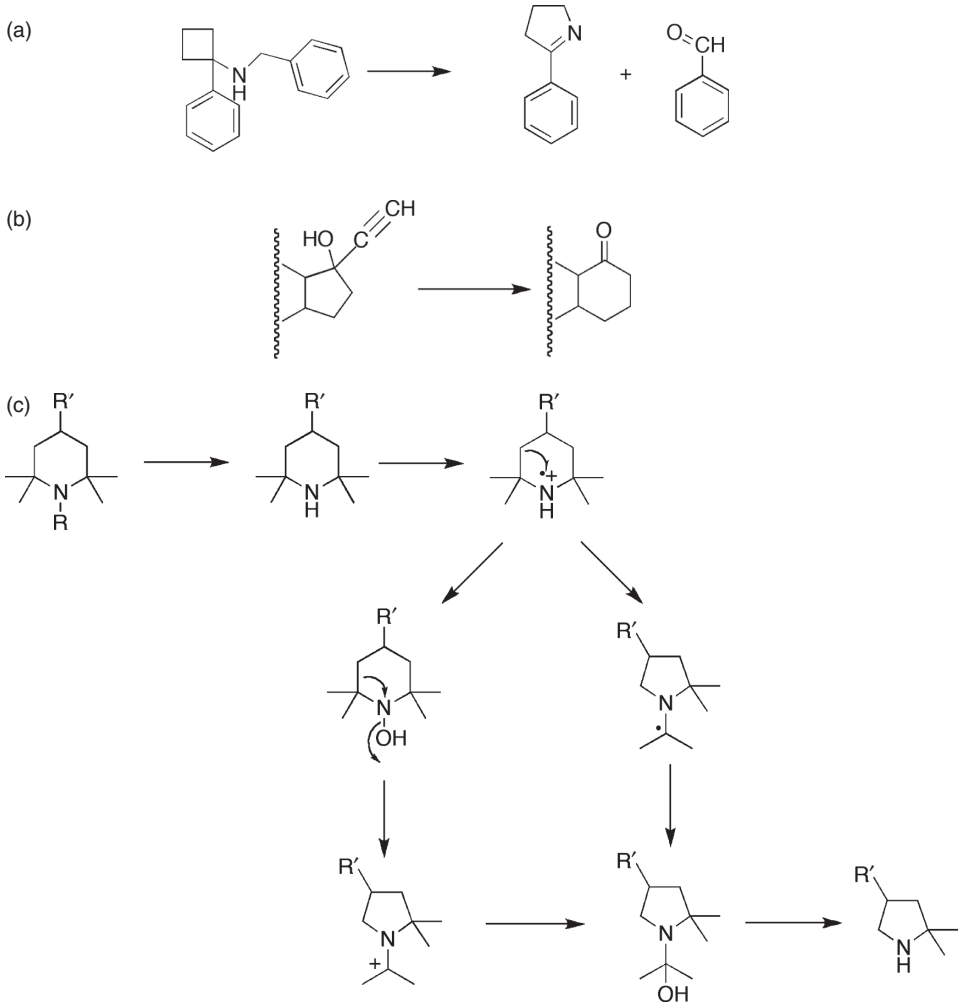

Figure 7.11 Some more "unusual" P450 reactions. (a,b) Ring expansions [104,125]. (c) Ring contraction [123]. (d) Ring formation [124].

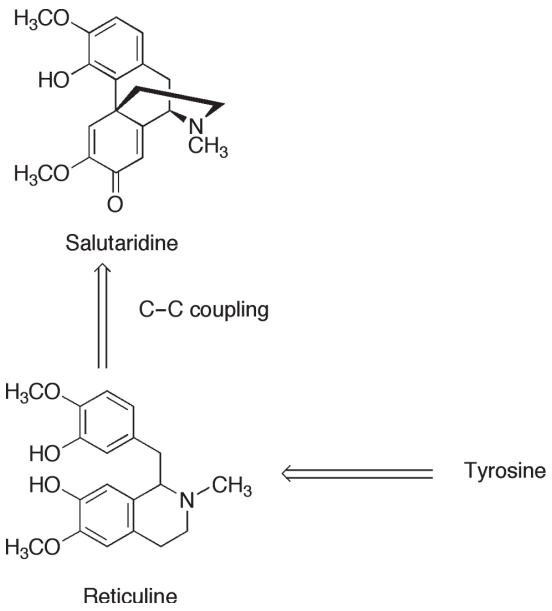

Figure 7.12 C–C bond coupling catalyzed by the P450s 2D6 and 3A4 [127].

7.4 SUMMARY

While reading about the details of P450 mechanisms, one can get the impression that some of the work is esoteric and unlikely to be of practical use. What is true is that some elements of P450 catalysis will be more practical than others. Following are several points that a drug metabolism scientist should remember and will be of use.

1. The basic sequence of events in the catalytic cycle is shown in Fig. 7.2. One should remember that the scheme is not necessarily ordered as such [10] and that which events are rate limiting can vary considerably among the P450s and substrates.
2. The general concepts of abstraction by FeO^{3+} and oxygen rebound will explain most P450s, so mastering this basic chemistry is important.
3. Rearrangements are to be expected, either before the oxygen rebound or after product formation, so these possibilities should be remembered in considering how a product might be formed.
4. P450 regioselectivity is driven by thermodynamic and steric aspects of possible P450 reactions, which are not independent of each other. The chemical reactivity at a given site can be predicted, but even with the available P450 crystal structures, predictions are difficult. Predictions about regioselectivity—and particularly rates—are most feasible in a series of compounds where data already exist for some of the members.

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8 Transcriptional Regulation of Cytochrome P450 Genes

EMILY A. FRASER, COLIN J. HENDERSON, and C. ROLAND WOLF
Division of Cancer Research, University of Dundee, Medical Research Institute, Ninewells Hospital & Medical School, Dundee, UK

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8.1 INTRODUCTION

Our mechanistic understanding of the transcriptional regulation of cytochrome P450 (P450) expression has increased significantly in recent years, with the discovery and characterization of "orphan" nuclear receptors such as the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR). The role of these receptors as mediators of the inductive effects of xenobiotics continues to be the subject of intensive investigation and has added a further layer of complexity to an already burgeoning area of research.

It has become clear that significant variability exists at almost every level of the processes that contribute to the ultimate functionality of P450 proteins, and transcriptional regulation is no exception. Expression of these proteins is controlled by transcription factors that not only vary in their expression level and the degree to which they bind and respond to different classes of ligands but are also themselves subject to alternative splicing, producing variant receptor forms with different spatiotemporal expression and functionality. It is clear that only by gaining a detailed understanding of these receptors will we have a greater appreciation of the intricacies of transcriptional regulation of P450s.

This chapter concentrates on those transcription factors involved in xenobiotic-induced expression, giving an overview of regulatory interactions and raising issues that have a bearing on transcriptional regulation, such as polymorphisms, splice variants, and species specificity. However, it is important to note that other transcription

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factors that are not covered in this review - including the peroxisome proliferator activated receptors α and γ, the vitamin D receptor, and the oestrogen receptors α and β - are also involved in regulation of endogenous and basal cytochrom P450 expression.

8.2 TRANSCRIPTIONAL REGULATION OF CYTOCHROME P450s

8.2.1 Pregnane X Receptor (PXR)

The pregnane X receptor (PXR, also known as SXR, PAR, and NR1I2) is the most recently identified of the three main regulators of xenobiotic-induced CYP expression, with the mouse ortholog having been first identified by Kliewer *et al*. in 1998, closely followed by the identification of the human form by Blumberg *et al*. [1,2]. A ligand-activated orphan nuclear receptor was named the pregnane X receptor because of its activation by pregnenolone derivatives. Acting as a xenosensor, PXR is one of the key controllers of drug metabolism, in particular, for the expression of the CYP3A enzyme isoforms. In addition to the CYP3A enzymes, PXR, bound as a heterodimer to its partner, the retinoid X receptor (RXR), regulates a wide variety of P450s, as well as phase II enzymes and phase III drug transporters (Table 8.1). PXR is also promiscuous, being able to accommodate and be activated by a wide range of different ligands, both endogenous and exogenous (Table 8.2). This protein is expressed in many tissues, including those of heart, colon, stomach, and certain brain regions, although it is primarily expressed in the liver and small intestine [3]. Several splice variants have also been identified, which affect PXR-mediated transcriptional regulation (Section 8.3.3).

Flexibility in the ligand-binding domain (LBD) of PXR is the reason for its wide substrate specificity, with the binding pocket able to expand from its resting 1150 $Å^3$ to more than 1600 $Å^3$ when ligand bound. Large molecules, such as the macrolide antibiotic rifampicin (RIF), can therefore activate the receptor using an induced-fit

TABLE 8.1 Examples of Human Cytochrome P450s Regulated by Human PXR, CAR, and AHR

Gene	PXR	CAR	AHR	References
CYP1A1	—	—	↑[a]	[4][a]
CYP1A2	↑	↑[b]	↑[a]	[5][b] and [4][a]
CYP1B1	↑	↔	↑	[6,7]
CYP2A6	↑[b]	↑	—	[8][b]
CYP2B6	↑	↑	↔	—
CYP2C8	↑	↑	—	[9]
CYP2C9	↑	↔	↔[b]	[10] and [4][b]
CYP2C19	↑	↑	—	—
CYP3A4	↑	↑	↔	—
CYP7A1	↓[b]	↓[a]	↔[c]	[11],[b] [12],[a] and [4][c]
CYP8B1	↓[b]	↓[p]	—	[11][b] and [13]
CYP24A1	↑	↑	—	[14]

Bold arrows indicate strong induction.

↑, induced; ↓, repressed; ↔, no change/basal expression; [p], putative interaction in humans. a, b, or c on each line refers change in nuclear receptor expression to the corresponding citation.

Source: Unless otherwise stated, data was extracted from Ref. 15.

TABLE 8.2 Example Modulators of Human PXR, CAR, and AHR

Class	Compound	PXR	CAR	AHR	References
Classical	Rifampicin	++			PXR/CAR: [7,15–21] AHR: [6,22]
	CITCO		++		
	TCDD			++	
	3-Methylcholanthrene			++	
Drug	Omeprazole	+		+	
	Thiabendazole			+	
	Nicotine			+	
	Caffeine			+	
	Paclitaxel	+			
	Methadone	+	+		
	Clotrimazole		−		
	Ketoconazole	−	−		
	17α-Ethinylestradiol	+			
	Mifepristone	+			
	SR12 813	+			
	Phenytoin	+			
	Primaquine	+			
	Spironolactone	+			
Herb	Hyperforin	++			
	Cryptotanshinone	+			
	Artemisinin	+	+		
	Ginkgo biloba	+	+		
	Schisandrin A-C	+			
Dietary	β-Carotene	+		+	
	Vitamin E	+			
	Flavonoids			±	
	Curcumin			+	
Endogenous	Androstanol		+		
	Corticosterone	+			
	17β-Estradiol	+	+		
	Vitamin K_2	+			
	Tryptophan metabolites			+	
	Bilirubin			+	
Environmental/ industrial	Benzyl butyl phthalate	+			
	Chlordane	+			
	Phthalic acid (DHEP)	+	+		
	Nonylphenol	+	+		
	Polychlorinated biphenyls	+			
	Toxaphene	+			
	Triclopyr	+			
	Benzo(*a*)pyrene			+	
	1-Methyl-1-phenylhydrazine			+	

Blank spaces indicate no data is available.

++, very strong agonist; +, agonist; –, antagonist (or inverse agonist, CAR only); ±, antagonist/agonist, dependent on cell context.

For further information see reviews [6,15–17].

mechanism, which allows molecules to adopt various binding orientations before selecting the optimal configuration [23–25]. The LBD is predominantly hydrophobic in character, with Met243, Ser247, Gln285, Trp299, His407, and Phe420 commonly interacting with ligands [26,27]. Site-directed mutagenesis studies have indicated that even small changes in the LBD can have significant effects on the ligand-induced activation of PXR [25,26]. Interspecies variability in this region therefore has significant effects on the ligand activation profile (Section 8.3.2).

Nuclear translocation is a controlling factor in PXR-mediated P450 induction. However, because of difficulties with investigating translocation *in vitro*, namely, when PXR is overexpressed it spontaneously translocates to the nucleus [28], data regarding this mechanism remains limited. It has been reported that when inactive, PXR is bound in a complex with cytoplasmic CAR retention protein (CCRP) and HSP90, which retains the receptor in the cytoplasm [29,30]. Although the mechanism underlying PXR dissociation from the complex and translocation to the nucleus has not yet been elucidated, it is likely that a mechanism similar to that governing CAR dissociation from the retention complex is involved (Section 8.2.2). Once released from the cytoplasmic retention complex, PXR nuclear localization signals (NLSs) are targeted by importin-α proteins for nuclear import [29].

Upon entering the nucleus, PXR heterodimerizes with its binding partner, the RXRα, before binding to response elements in the promoter and enhancer regions of target genes (Fig. 8.1). PXR heterodimers preferentially bind direct repeat sequences possessing the half site AG(G/T)TCA separated by three nucleotides (DR3) and everted repeats of the half site separated by six nucleotides (ER6), that is, AG(G/T)TCAnnnAG(G/T)TCA and TGA(A/C)CTnnnnnnAG(G/T)TCA, respectively [15,16]. However, they are also able to bind other recognition sites, such as DR4, DR5, and ER8 motifs, although with lower affinity [31], thus accounting for the cross talk between receptors, which is an important contributor to P450 regulation.

A key component of transcriptional activation by nuclear receptors is the recruitment of coactivators and corepressors to promoter regions, following DNA binding. Coactivators of PXR, CAR, and AHR (aryl hydrocarbon receptor) are given in Table 8.3. As an example, PXR is a binding partner for the p160/SRC (steroid receptor coactivator) coactivator family, which recruits histone acetyltransferase complexes, such as cAMP response element binding protein/p300 (CREB), thus providing access to DNA strands for RNA-polymerase-catalyzed transcription [25]. These coactivators possess three LXXLL motifs that bind to the AF-2 helix, interacting with two charged residues on the receptor surface (Lys259 and Glu427) to form a charge clamp [24]. Crystal structures of SR12813 in complex with PXR and SRC-1 indicate that in the presence of coactivator, the ligand will bind in a single orientation. The combination of ligand and SRC-1 binding stabilizes the protein structure to restrict the flexibility of the LBD once an active ligand conformation has been achieved [25]. In contrast, corepressors are thought to bind in the absence of ligand, or in the presence of antagonists, retaining the AF-2 helix in a nonactive conformation [16]. Corepressors of PXR include the nuclear receptor corepressor (NCoR) and the silencing mediator of retinoid and thyroid hormone receptor (SMRT). SMRT possesses both ID1 and ID2 interacting domains, which consist of a CoRNR box (I/LXXI/VI) and the motif LXXXIXXXI/L, respectively [32]. PXR binds to the ID2 motif preferentially over the ID1, with the key interacting residues being Lys259, Gly270, and Pro423 of the PXR LBD and Arg2347, Lys2348, and Leu2350 of SMRT [32]. Site-directed mutagenesis studies show that mutation

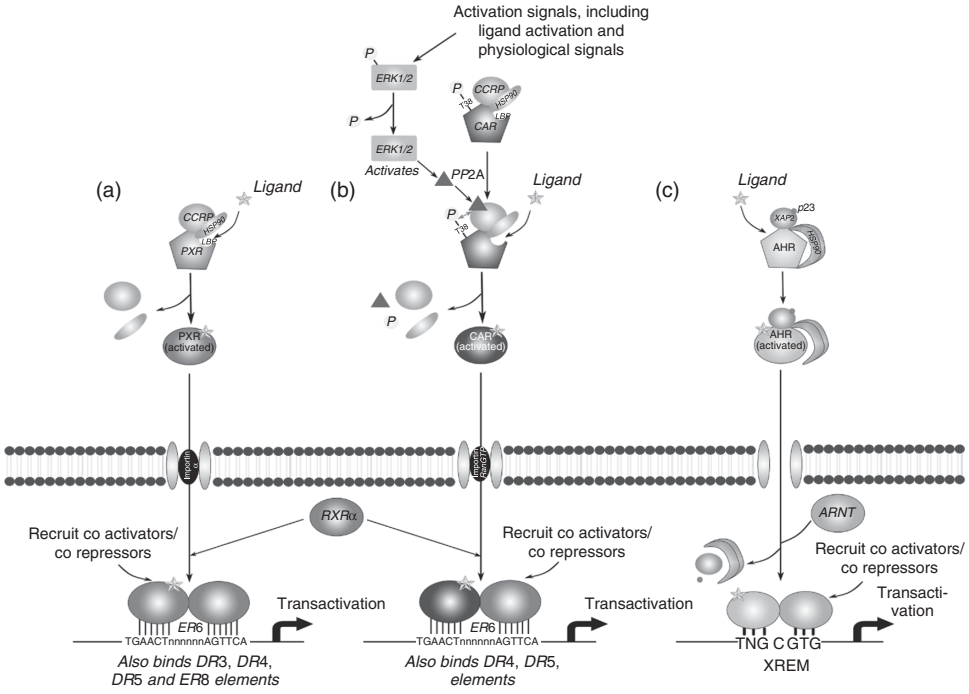

Figure 8.1 Activation mechanisms of (a) PXR, (b) CAR, and (c) AHR. (See color insert.)