HHS Public Access

Author manuscript

ACS Catal. Author manuscript; available in PMC 2019 May 15.

Published in final edited form as:

ACS Catal. 2018 December 7; 8(12): 10964–10976. doi:10.1021/acscatal.8b03401.

Mechanisms of Cytochrome P450-Catalyzed Oxidations

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Abstract

Enzymes are complex biological catalysts and are critical to life. Most oxidations of chemicals are catalyzed by cytochrome P450 (P450, CYP) enzymes, which generally utilize mixed-function oxidase stoichiometry, utilizing pyridine nucleotides as electron donors: NAD(P)H + O_2 + R → $NAD(P)^+$ + RO + H_2O (where R is a carbon substrate and RO is an oxidized product). The catalysis of oxidations is largely understood in the context of the heme iron-oxygen complex generally referred to as Compound I, formally FeO^{3+}, whose basis was in peroxidase chemistry. Many X-ray crystal structures of P450s are now available (≥ 822 structures from ≥146 different P450s) and have helped in understanding catalytic specificity. In addition to hydroxylations, P450s catalyze more complex oxidations, including C-C bond formation and cleavage. Enzymes derived from P450s by directed evolution can even catalyze more unusual reactions, e.g. cyclopropanation. Current P450 questions under investigation include the potential role of the intermediate Compound 0 (formally Fe^{III}-O_2^-) in catalysis of some reactions, the roles of high- and low-spin forms of Compound I, the mechanism of desaturation, the roles of open and closed structures of P450s in catalysis, the extent of processivity in multi-step oxidations, and the role of the accessory protein cytochrome b_5. More global questions include exactly how structure drives function, prediction of catalysis, and roles of multiple protein conformations.

Graphical Abstract

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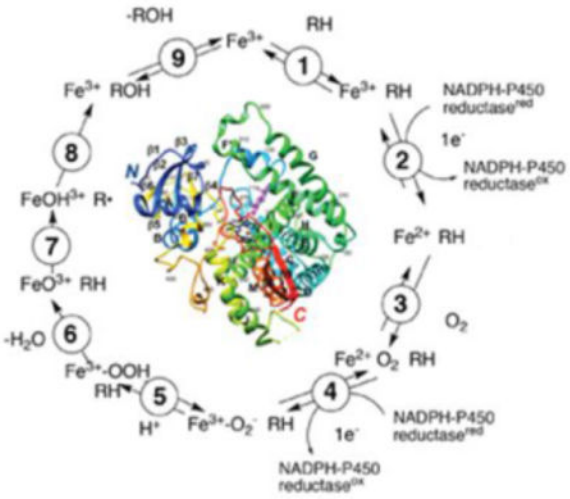
Conflict of Interest. The author declares no conflict of interest.



Keywords

Cytochrome P450; enzymology; cytochrome b_5; Compound I; directed evolution; processivity; kinetics; oxidation

Background and Introduction

Catalysts come in many forms, and enzymes are the catalysts in most biological reactions. Like all catalysts, they are not consumed in reactions. Enzymes can control regioselectivity and stereospecificity of reactions of complex organic molecules and, because they are chiral catalysts (i.e., built of L-amino acids), they can induce stereoselectivity into seemingly non-chiral molecules (e.g., distinguish between pro-*R* and pro-*S* hydrogens on a simple methylene group such as that in ethanol[1,2]). Unlike most other catalysts, enzymes are synthesized in biological systems, coded for by genes.

The functions of enzymes vary, and several types of enzymes catalyze oxidations and reductions.[2] Of these, ≥ 95% of those reported in the literature are done by cytochrome P450 (P450, CYP) enzymes.[3] Most of the P450 reactions are oxidations and involve the use of molecular oxygen, i.e. O_2. P450s almost always act as monooxygenases, or mixed-function oxidases, using the stoichiometry shown in Equation 1 and utilizing the pyridine nucleotide NADH or NADPH as a cofactor (used to deliver electrons via a flavoprotein, sometimes via an iron-sulfur protein)

$$\mathrm{NAD(P)H + O_2 + R \rightarrow NAD(P)^+ + RO + H_2O} \quad (1)$$

(where R is an organic substrate and RO is the reaction product), as opposed to the stoichiometry used in dioxygenases (Equation 2)

$$\mathrm{O_2 + R \rightarrow RO_2} \quad (2)$$

or (Equation 3)

$$O_2 + R + R' \rightarrow RO + R'O \quad (3)$$

where both oxygen atoms are incorporated into organic substrates.[2,4–6]

An important concept to remember in catalysis involving oxygen is that O_2 is in the triplet state and its direct reaction with singlet organic molecules is spin-forbidden.[5–7] Metalloproteins such as P450s overcome this barrier by complexing the oxygen to a metal (iron), so that the metal-oxygen complex can react with carbon substrates, as well as some heteroatoms and even metals.[8]

The first reports of P450 were published in 1962 and 1964.[9–11] Before that time, the concepts of dioxygenases and mixed-function oxidases had been established by Hayaishi, Mason, and others.[4,12,13] The concept of generating oxygenated compounds by activated oxygen species was in contrast to earlier views by Wieland and others that these alcohols and other oxygenated chemicals were generated from the activation of carbon and subsequent reaction with water.[6,12–14] The connection between P450 and mixed-function oxidation (of a steroid) was made later by Cooper *et al.*[15] using photochemical action spectra; i.e. light reversal of inhibition of a hydroxylation reaction by carbon monoxide.

Subsequent work with bacterial and mammalian systems led to the purification of many P450s. Early proposals about the generation of mobile oxygen species and their roles[16,17] have been abandoned in favor of high-valent iron oxygen complexes that do the oxidation chemistry, overcoming the problem of spin-forbidden reaction of singlet (carbon) substrates with triplet oxygen and restricting the regioselectivity of the reaction to a specific site(s) on a substrate.[2,5,6] The oxygenated forms of P450 enzymes have considerable similarity to those used by other iron-based monooxygenases and dioxygenases.[6,18]

Current Understanding

The catalytic cycle shown in Scheme 1 is generally accepted for most P450- catalyzed oxidations. The intermediates between steps **4** and **8** are unstable and have been difficult to characterize. There is some ambiguity in the exact location of the electronic charge in these entities, and variations are seen in the literature, i.e. in the distribution of charge in the iron atom, the Fe-O bond, and the porphyrin ring.

Most P450 oxidations are considered to occur via Compound I, the formal FeO^{3+} entity shown in Scheme 1 (i.e., steps **7** and **8**), named because of its history in peroxidase chemistry.[20] Much of our understanding of the catalysis came from work on biomimetic models; i.e. model metalloporphyrin oxide complexes.[21,22] Compound I has been characterized in some bacterial P450s by Green and associates[23–25] and by Makris and associates.[26] Compound I abstracts a hydrogen atom from an alkyl group (e.g., methyl, methylene) yielding a carbon radical (step **7** in Scheme 1). The formal Fe-OH complex reacts rapidly with this radical to generate the product, often an alcohol, in a so-called “oxygen rebound” step (step **8** in Scheme 1). In cryo-annealing/γ-ray reduction studies by

Hoffman and Sligar, it has been possible to characterize dioxygen intermediates of P450 101A1 ($P450_{cam}$) and P450 11A1 by ENDDR spectroscopy and to document catalytic competence as judged by product formation.[27–29]

At this point it should be mentioned that many X-ray crystal structures are now available (≥ 822 structures for ≥ 146 different P450s currently in the Protein Data Bank, with 112 uploaded but not yet released as of the date of revision of this manuscript). These have generally similar folds but the size and shape of the active site vary considerably, yielding catalytic specificity among P450s.[30] Most of the structures are of ferric enzymes, many with substrates or inhibitors present. Some structures of ferrous and ferrous-oxygen complexes of bacterial P450s 101A1 ($P450_{cam}$) and 107A1 ($P450_{eryF}$) are available (e.g., PDB 1DZ8, 1T88, 2A1M, 2A1N, 2A1O, 1Z8O, 1Z8P, 1Z8Q)[30–33]

Not all P450 reactions are carbon hydroxylations, but Compound I is involved in these as well. Compound I has a high oxidation-reduction potential and, when in the proper position, can abstract electrons from amines[34–36] and other relatively low potential reductants, e.g. certain strained alkanes and substituted aromatic ring systems.[37,38] The abstraction of an electron from an amine is the initial step in many *N*-dealkylation reactions.[19,34,39,40] With some of these amines and also carbon substrates, the carbon radical generated in step **7** (Scheme 1) can rearrange before step **8** occurs,[41] even though the rebound reaction is generally fast. This competition between oxygen rebound and rearrangement can be "clocked" with strained alkanes having known rates of rearrangement in solution.[42,43] Other competing reactions with Compound I are reduction to water[44] and abstraction of a phenolic hydrogen atom from a (P450) tyrosyl group, which confounded early efforts to characterize Compound I.[25,45–48]

For more extensive lists of rearrangements and more unusual oxidation reactions catalyzed by P450s, see references 6, 8, 19, 49, 50. Most of these can be rationalized by mechanisms involving Compound I, with Compound 0 (Scheme 1) possibly involved in others.

Current Questions

Compound I vs. Compound 0.

Compound 0 (formally Fe^{III}-O_2^- or its protonated form) has been invoked in some P450 reactions that have been difficult to explain with Compound I.[8,19] The difficulty in discriminating between Compound 0 and Compound I derives from instability of these species and the transformation of Compound 0 to Compound I in the catalytic cycle (Scheme 1). Any effort to discern these by site-directed mutagenesis of a P450 has the caveat that other aspects (not only proton transfer) have been modified. Compound I is an inherently electrophilic species and (unprotonated) Compound 0 is nucleophilic. The use of "oxygen surrogates" can be useful, with appropriate caveats, e.g. iodosylbenzene (and alkyl hydroperoxides) can generate Compound I (or a closely-related structure[51,52]) but cannot produce the dioxygenated complex Compound 0 (Scheme 1).

One of the historically dominant pieces of evidence supporting a role for Compound 0 was $^{18}O_2$ labeling work with P450 19A1, in which one atom of ^{18}O was reported to be

incorporated in the product formic acid in the third and final step of the steroid aromatase reaction.[53–55] Conclusions about the mechanism were extrapolated from this result to other P450 reactions.[56] However, when the original experiments with P450 19A1 were repeated under more technically sophisticated conditions, no ^{18}O incorporation was observed and the results are only consistent with a role for Compound I and not Compound 0.[6,57]

Another P450 C-C cleavage reaction, the periodate-like cleavage of 20,22-dihydroxycholesterol to two carbonyl products, is done by P450 11A1 Compound I, as demonstrated by a relatively high yield (~ 50%) of product formed by radiolytically-generated Compound I.[29]

The case of the P450 17A1-catalyzed cleavage of 17α-hydroxy steroids ("lyase" reaction) is more problematic, in that the documented incorporation of one atom of ^{18}O[58] has been repeated[52] but this result is not unambiguous regarding the mechanism. Raman spectral evidence for a Compound 0 species has been generated in the presence of substrate but this entity has not been shown to be catalytically competent (i.e., converted to product).[59–61] Iodosylbenzene and 17α-hydroperoxy steroids (oxygen surrogates) both yield the 19-carbon lyase reaction products,[52,62] but the involvement of Compound 0 in the normal reaction cannot be ruled out.[6]

The purported roles of Compound 0 in other reactions remain to be investigated, e.g. lanosterol 14 α-demethylation (P450 51 species)[63] and the cleavage of nabumetone.[64,65]

Multiple States of Compound I.

Theoretical calculations, primarily by Shaik and his associates, have largely discounted all potential oxygenated intermediates other than Compound I in the catalytic cycle (Scheme 1) as unable to be involved in P450 oxidations, including Compound 0 and Compound II (formally $FeOH^{3+}$, following step **7** in Scheme 1).[66] Theory has also led to the concept of "two-state reactivity," i.e. the view that different forms of Compound I are involved in different reactions, even with a single substrate. This subject (which is too complex to fully consider here) is often approached in the context of "high-spin" and "low-spin" states of Compound I and their reactivity, but the possibilities for electronic distribution and multiple forms of Compound I are more complex. Shaik and associates use the term "chameleon nature" of Compound I, meaning that Compound I can change its character depending upon its environment. That is, the juxtaposition of a substrate in the enzyme can shift the balance of states of Compound I and affect the mechanism.[66]

The concept has attraction in explaining multiple reaction courses. For instance, epoxidation of aryl compounds is observed along with formation of phenols and ketones, but the epoxide does not necessarily form the products nor does a Meissenheimer complex necessariily form epoxides. Epoxidation of olefins is often accompanied by 1,2- shifts indicative of cationic intermediates (plus heme destruction).[67–69] These sets of reactions have often been attributed to a single P450 intermediate that collapses in different ways (Scheme 2A)[8,70] but the chameleon theory would suggest different electronic distributions of Compound I participating in formation of different products (Scheme 2B).

This concept sounds very reasonable but unfortunately it is not very possible to prove experimentally (unless one considers more theoretical work experimental). The theoretical considerations are based largely on rationalization of prior experimental data, and development of new predictions that could distinguish among alternate mechanisms is difficult. In principle, the barrier to one reaction could be raised and the result observed, in terms of product redistribution (Scheme 2). However, the result could be explained by either (i) shifting the positioning of the substrate in the active site to favor one reaction (due to substrate motion) or (ii) to the ability of alternate forms of Compound I to do the reaction (at the same site in the protein). Although bond strength is one issue in influencing product reactivity (e.g., the preference of allylic sites for hydroxylation[71]), it is not the only one and examples of deficiencies in prediction are considered later *(vide infra,)*.[72]

In the author's opinion, these issues may ultimately be addressed experimentally in studies with artificially-generated Compound I *(vide supra)*, in which detailed spectral analysis and product measurements are done.[23–26,29]

Open vs. Closed Structures.

More crystal structures (≥ 119) of bacterial P450 101A1 ($P450_{cam}$) are available than for any other P450. These structures, with the substrate camphor and other ligands (e.g., PDB 2CPP, 7CPP, 1PHA, 1PHB, 1PHC, 5IK1), can be grouped into three conformational states: open, closed, and intermediate.[30,73–76] The states involve differential movements of the F and G helices that cover the substrate binding channel. The studies support a view that this protein exists in a small set of distinct conformations, rather than a more continuous distribution of malleable, induced-fit states.[76] How this paradigm applies to mammalian and other eukaryotic P450s is unknown, although there is considerable evidence that those enzymes also have at least open and closed conformations.[30]

One issue is the effect of the auxiliary protein putidaredoxin on the structure of $P450_{cam}$. Putidaredoxin has long been known to be the immediate electron donor to $P450_{cam}$,[77] but it has also been known for many years to have an additional "effector" role in catalysis, in which it enhances product formation in a role separate from electron delivery.[78] Although this role has been known since 1972,[78] the exact mechanism has remained unclear. The general consensus is that oxidized putidaredoxin remains bound to the reduced P450 $Fe^{2+}O_2$ (or Fe^{3+}-O_2^-) complex long enough to alter its conformation and facilitate steps **5**–**8** of the catalytic cycle (Scheme 1). Several structural studies have provided contrasting answers to how this happens. A crystallographic study with a crosslinked $P450_{cam}$-putidaredoxin complex indicated that $P450_{cam}$ was in the open form (with product bound),[79] as did an independent double-electron-electron resonance (DEER) study with spin labels attached to $P450_{cam}$.[80] However, an NMR complex yielded an opposite result, i.e. a closed complex with $P450_{cam}$.[81]

These studies leave questions open about the catalytic mechanism, and the exact relevance to most eukaryotic P450s is unknown. Further DEER studies on $P450_{cam}$ with different spin labels also show that oxidized putidaredoxin induces reduced $P450_{cam}$ to change to the open form.[76] Carbon monoxide binding somehow prevents this shift. Further DEER measurements have indicated that putidaredoxin binds to the same site of $P450_{cam}$ in both

the open and closed forms.[82] Apparently this binding triggers the conformational change through rather subtle structural interactions.[82] Exactly how this information is relevant to the human P450 redox partner interactions is still unclear (i.e., NADPH-P450 reductase and cytochrome b_5 (b_5) in the microsomal P450s and adrenodoxin in the mitochondrial P450s).

Desaturation.

Numerous examples of P450-catalyzed desaturations are known, and the process does not involve dehydration of an alcohol product.[8] In many cases, the desaturated product is minor relative to the alcohol, consistent with the view that a common intermediate (step **7**, Scheme 1) leads to both.[83–85] In several cases,[86–90] including fish and human P450s,[91,92] the major or only product is the desaturated product.

Presumably the reaction involves the abstraction of a second hydrogen atom in the $FeOH^{3+}$ radical intermediate (Schemes 1, 3). Whether this is actually a hydrogen atom abstraction or proton-coupled electron transfer is not clear. Site-directed mutagenesis work with (non-heme) iron-based fatty acid desaturases and the iron/α- ketoglutarate dioxygenase AsqJ indicates that the balance between desaturation and hydroxylation is rather sensitive to minor changes,[86,93–95] and P450s are probably similar in this regard (Scheme 3).

Processivity.

Many P450 reactions involve multiple steps, i.e. a product of one reaction is a substrate for a subsequent reaction by the same enzyme. An issue is the degree to which the two reactions are “processive,” i.e. the extent to which the first product is released by the enzyme and must re-bind to generate the final product. If a reaction is not processive, it is “dissociative” (Scheme 4). The balance is not simply a matter of kinetic curiosity, but the coupling of reactions (i.e., degree of processivity) is an indication of whether a biological process is deterministic or stochastic. A more deterministic sequence of reactions may be important, in terms of biological function. The nature of the processivity can also be an issue when considering development of enzyme inhibitors.[96,97]

This issue has been investigated in several cases involving steroid oxidations.[97–102] The design of such experiments is critical, and pre-steady-state kinetic approaches are necessary.[103] Our own work led to the conclusion that the three steps in the P450 19A1 steroid aromatase reaction are distributive.[102] Studies on animal and human P450 17A1 reactions (steroid 17α-hydroxylation and subsequent C17 α,20 cleavage (lyase reaction)) indicate partial processivity,[97–101] although only in the case of the human P450 17A1 studies was the highly stimulatory protein b_5 included.97

Pulse-chase experiments indicated that the P450 2E1-catalyzed oxidations of ethanol and *N*,*N*-diethylnitrosamine to acetic acid are both processive, with the latter being less processive than the oxidation of *N*,*N*-dimethylnitrosamine to formic acid (Scheme 4).[105,106] P450 2A6 also showed processivity in the same oxidation of the above two nitrosamines to 1- and 2-carbon carboxylic acids.107 However, in these reactions the results were not fit to quantitative models with rate constants for both product dissociation and the second

oxidation step. These situations have a dilemma in that there was no evidence for high-affinity binding of acetaldehyde or formaldehyde.

Other P450s known to catalyze sequential oxidations with endogenous chemicals include 11A1, 11B1, 11B2, 24A1, 27A1, 46A1, and 51A1. Human P450s catalyzing sequential reactions with xenobiotics include 1A1, 1A2, 1B1, 2A6, 2A13, 2C9, 2E1, and 3A4.

Role of b_5.

b_5 is a microsomal heme protein that has an electron transfer role in fatty acid desaturation and some other enzymatic reactions.2 In 1971, Estabrook and Hildebrandt108 postulated that b_5 could be involved in P450 reactions in rat liver microsomes, on the basis of results of stimulation with the cofactor NADH, which only interacts poorly with NADPH-P450 reductase to reduce P450s but reduces NADH- b_5 reductase and then b_5. With the purification and reconstitution of P450s and b_5, a number of results have been reported. Depending upon the system, investigators have reported either no effect, stimulation, or inhibition with the addition of b_5.[109–112] Even with a given P450, the results can vary depending upon the reaction under consideration.[110,113]

The original postulate was that b_5 is transferring the second electron (to the $Fe^{2+}O_2$ (or $Fe^{3+}-O_2^-$) complex, step **4** in Scheme 1).108 However, with several P450s either apo-b_5 (devoid of heme) or Mn^{2+}-substituted b_5 can stimulate as well as holo-b_5 (with the normal heme).[114–117] A proposal that heme is transferred from the P450s to the apo-b_5 during the course of enzyme reactions[118] was shown to be untenable.[119]

At least four mechanisms for b_5 stimulation have been proposed: (i) electron transfer (to the $Fe^{2+}O_2$ (or $Fe^{3+}-O_2^-$) complex, step **4**, Scheme 1); (ii) an allosteric effect that favors a more active conformation of the P450s; (iii) an effect of decreasing the abortive use of electrons to generate H_2O_2, avoiding uncoupling; and (iv) facilitating the protonation of the hydroperoxo intermediate (step **5** in Scheme 1). Waskell and colleagues have provided EPR evidence for the latter proposal in the case of rabbit P450 2B4-catalyzed *d*-benzphetamine *N*-demethylation,[120] although this reaction is not strongly influenced by the presence of b_5 under all conditions.[110] Enhanced protonation of the peroxo complex would actually be inhibitory to the strong stimulation of the lyase activity of the P450 17A1 if the proposed nucleophilic Compound 0 mechanism[58–61] is viable, in that decomposition to Compound I would be facilitated. Apo-b_5 can stimulate reactions as well as b_5 with several P450s[114,117,119] but is ineffective with some P450 reactions,[117,121–124] thus implicating electron transfer in those.

There are several experimental deficiencies in the b_5 field. Clearly b_5 must bind to P450s, as shown by NMR perturbation[125] and crosslinking[126] studies, but estimates of binding parameters have only been made in two cases, and then by surface plasmon resonance,[127,128] which can be notoriously inaccurate due to surface artifacts.[104] No crystal structures of b_5:P450 complexes are known (other than computational). Also, kinetic investigations with b_5 and high-valent P450 intermediates are limited because b_5 must be preincubated with P450 in vesicles (i.e., unlike a ferredoxin it cannot be added quickly in a stopped-flow experiment). NMR investigations by Estrada and Scott[125] also indicate that b_5 and NADPH-

P450 reductase compete for binding at the same site of P450 17A1, precluding some experimental kinetic designs (see also ref. 129).

To add to the complexity are *in vivo* transgenic mouse experiments.[124,130] Conditional deletion of microsomal b_5 in liver creates a liver b_5-null mouse, but these mice develop and breed normally and have no overt phenotype. In liver microsomes prepared from these animals, NADH-mediated metabolism was essentially abolished for most substrates, and the NADPH-dependent metabolism of many substrates was reduced by 50–90%131 (the NADH-b_5 reductase/b_5-supported reduction of P450s can be reconstituted *in vitro*, despite the unfavorable redox potential of b_5 for reducing ferric P450s (step **2** in Scheme 1)[123,132]). The results indicate that microsomal b_5 can play a major role in the *in vivo* metabolism of certain drugs and chemicals but in a rather P450- and substrate-dependent manner.130 The extent to which metabolism was significantly affected by the (hepatic) absence of b_5 is substrate-dependent.131 In mice treated with the carcinogen benzo[*a*]pyrene, *in vivo* DNA adduct levels were significantly higher (7-fold) in the livers of (liver) NADPH-P450 reductase knockout mice than wild-type mice.124 In the same study, no significant difference in DNA adduct formation was observed in liver between NADH-b_5 reductase-null and wild-type mice. Thus, NADPH- P450 reductase and b_5 both appear to modulate P450-mediated activation of benzo[*a*]pyrene *in vitro* but hepatic P450 enzymes appear to be more important for benzo[*a*]pyrene detoxication than its activation *in vivo*.

What are the Limits of P450 Catalysis?

Recent work by Arnold and associates[133–137] and also by the Fasan group[138–145] has pushed the limits of what P450s—and other heme proteins—can do in the realm of catalysis. Arnold's work in directed evolution, which was recognized by a share of the 2018 Nobel Prize in Chemistry, includes ≥ 47 papers with P450s and their derivatives.

This area developed largely from work with bacterial P450 102A1 (P450bm3), which has advantages over most other P450s in that it is self-sufficient (i.e., it has a reductase domain that binds NADPH and delivers electrons to the heme domain, eliminating the need to co-express a separate reductase), high catalytic activity towards fatty acids (turnover number ~ 200 s^{-1}), and high expression levels.[146] Directed evolution (or, more appropriately "molecular breeding"), with high throughput selection methods, was used to develop modified P450 102A1 catalysts that can catalyze various transformations of interest.[137] Some of the reactions involve drugs, and batteries of mutated P450 102A1 catalysts can be used to screen for their abilities to synthesize drug metabolites and new lead molecules.[147]

Further studies led to the ability to catalyze propane 1-hydroxylation, a dramatic departure from the classical $\omega-1$, $\omega-2$, … hydroxylation of fatty acids.[148] Another development was the synthesis of anti-Markovnikov carbonyl products from styrenes by selected P450 102A1 mutants (Scheme 5A),[135] although it should be noted that such reactions had been demonstrated earlier with rat P450 2B1[68] and with P450 biomimetic models.[149]

Further molecular breeding, incorporating random and site-directed mutagenesis and unnatural amino acid mutagenesis, has yielded P450-derived enzymes that can catalyze unusual reactions, e.g. cyclopropanation and nitrene transfer (Scheme 5B,5C).[133,134,143]

These reactions do not involve the normal P450 chemistry (Scheme 1), and Arnold's group found that substitution of the conserved cysteine heme ligand by serine led to higher activity.137 These catalysts are no longer P450s, by definition (no thiolate ligand or classical Fe^{II}-CO spectrum) and have been termed P411 by Arnold.[137,150] Nevertheless, some of these P411 variants are capable of highly stereoselective catalysis.134

The nitrene transfer mechanism (Scheme 6),144 with roots in earlier work by White151 and Dawson and Breslow,152 is rather unrelated to the normal catalytic cycle (Scheme 1). Consistent with the P411 catalysis, these reactions can also be catalyzed by myoglobin derivatives.[140]

Further Unresolved Issues

Exactly How Does Structure Drive Function?

At least 822 P450 structures (from > 146 P450s) are available in the Protein Data Bank, and a large fraction contain bound ligands. In many cases, the atom(s) on the substrate that is oxidized is positioned closest to the heme iron and, presumably, the oxygen of the FeO^{3+} complex (Compound I). Such structures can explain many P450 reactions, and it is certainly easier to understand reactions with enzyme structures than without any.

However, there are some gaps in the field, aside from the difficulty in crystallizing some of the P450s. As pointed out earlier, there is a paucity of structures of complexes with accessory proteins. Examples are with ferredoxin-P450 fusion proteins, one a mitochondrial P450 (11A1)[153] (PDB 3N9Y, 3NA1, 3N9Z, 3NA0, 4JWS, 4JWU, 4JX1, 3W9C) and two others with bacterial P450 101A1.[79,154] The only other case involves the separated heme and flavin domains of bacterial P450 102A1 (1BVY).[155]

Another issue is that the structures can explain major sites of reaction, but structures explaining minor products are not generally seen due to the energetic differences in binding in the less favorable conformations. In this regard, the computational prediction software may even be more generally useful than structural models.

Another issue is that there are some examples of explaining natural human (or other) genetic variants at a structural level but not many (e.g., PDB 5X23, 5X24).[156] For instance, it would be interesting (and useful) to understand why the human P450 2D6*53 variant has higher activity than wild-type P450 2D6.[157] Two recent examples from our own group exemplify the problems. Zebrafish P450 17A1 and P450 17A2 both catalyze steroid 17α-hydroxylations (progesterone and pregnenolone) but only P450 17A1 will catalyze the second (lyase) reaction, i.e., cleaving the C_{17}-C_{20} bond *(vide supra)*.[158] However, the X-ray structure showed nearly identical active sites,[158] and site-directed mutagenesis of all five possible perturbations (four near the heme periphery) did not impart lyase activity to P450 17A2.[62] In the case of human P450 21A2, > 100 single amino acid substitutions have been reported, with clinical deficiencies. Although having the structure of wild-type P450 21A2 is useful in understanding the low-activity variants,[159–161] we have been unsuccessful in obtaining diffractable crystals of any of the variants.

The difficulty of relating structure to function can be appreciated by considering the implications of the Eyring equation:

$$k_{obs} = \frac{k_B T}{h} e^{-\frac{\Delta G^{\pm}}{RT}} \quad (4)$$

, in which a difference of 1.3 kcal mol^{-1}, less than a typical single hydrogen bond, translates to a 10-fold difference in reaction rate at 37 °C (K_B is the Boltzmann constant, h is Planck's constant, and T is the absolute temperature). Further, a free energy difference of 6.4 kcal mol^{-1} is associated with a 50,000-fold change in the rate. Thus, small differences in structure (binding) can lead to major changes in catalysis. This, of course, is an issue not only for P450s but for all efforts at understanding enzyme structure-function relationships.

Prediction of Catalysis.

As mentioned earlier, structural models have the capability of understanding regioselectivity of oxidation reactions and even predicting sites with new ligands. The experience to date with AutoDock programs[162,163] is not better than with programs such as MetaSite[164–166] that are based on past history with similar molecules, in the absence of structural constraints. Success rates of ~ 80% in predicting the top three sites of oxidation are reasonable and competitive with predictions by trained drug metabolism experts.[167,168]

There are still anomalies that would not be predicted. For instance, testosterone and the important androgenic derivative dihydrotestosterone differ only in a double bond and twists in the (steroid) A-ring. However, P450 3A4 hydroxylates testosterone at the 6β position (plus lesser amounts of hydroxylation at the 2β, 1β, and 15β positions)[71,169] but dihydrotestosterone is hydroxylated primarily at the thermodynamically unfavored C18 methyl group.[72]

Another deficiency, especially in the field of drug metabolism, is that we do not really have the ability to predict rates of oxidation of compounds, even when we can predict sites. This can realistically only be done in situations where Hammett analysis (with substituted aryl groups)[170,171] or some other well-defined linear free energy or another quantitative structural-activity paradigm can be applied. Beyond such cases we have limited predictably with new substrates, as well as with predicting the effects of most single-amino acid substitutions in P450s. As noted above, the problem can be appreciated when the Erying relationship is considered.

Multiple Conformations.

One of the reasons why structural predictions are not better is the presence of multiple conformations of P450 proteins. There is considerable evidence for their existence, both from the structures of proteins bound to different ligands[30,96,172] and from the existence of multiple crosspeaks in NMR spectra.[173] In principle, NMR methods would be more useful in revealing multiple conformations but solving structures is formidable for such large proteins. Through-space spectroscopic methods such as fluorescence and EPR tagging are

qualitatively useful but also deficient in revealing total individual structures (and necessarily require perturbation of the protein). Molecular dynamics simulations, although more theoretical than experimental, can also address questions about multiple configurations.

The existence of multiple conformations of P450s, or at the very least multiple docking models, is self-evident in considering multiplicity of reaction products from a single enzyme and substrate. There is also extensive evidence for substrate-induced conformational changes upon binding, as clearly evidenced in the crystal structures[30] (and NMR measurements[125,173]).

One question is whether these changes involve induced fit or conformational selection models (Scheme 7), a general issue in enzymology.[174] In the induced fit model, the binding of the substrate leads to a conformation change. In contrast, in the conformational selection model, multiple states of the unbound protein coexist, one of which has substrate complementarity and binds (Scheme 7). Which of the two mechanisms is dominant with P450s is unclear, although some of our own kinetic studies with P450 3A4 led to a preference for an induced fit model.[175] The two phenomena are not mutually exclusive, and we have utilized two forms of both free and bound P450 to obtain fits of ligand binding kinetic data.[97,102,175,176] However, the improved fitting seen with adding more steps may be misleading, in that the most minimal kinetic models are generally the safest even if not the most complete.[103]

We have explored the effects of substrate binding on circular dichroism spectra. However, any changes seen are probably too weak to be useful, in that changes in helicity are needed.

Another anomaly of substrate binding is the slow changes in heme perturbation spectra seen with some P450s. Bacterial P450 101A1 binds its substrate (camphor) rapidly ($k = 10^6$ $M^{-1}s^{-1}$)[177] and so do several human P450s.[102,121,122,159] However, under the same conditions several mammalian P450s show slow changes in heme perturbation spectra upon mixing with substrate.[92,175,176,178,179] In some cases the rates of initial contact of the substrate/ligand with the P450 has been shown to be fast, as judged by fluorescence kinetics of interaction of ligands with the protein.[102,175,179] The slow heme perturbation results are currently rationalized in models that involve diffusion- limited encounters of a substrate with a ligand followed by conformational changes and “worming” of the ligand into the active site, moving near the heme iron.[179] However, it is presently unclear why some P450s exhibit such behavior and others do not, as well as whether an induced fit or a conformational selection model is more relevant.

Conclusions

P450 is a mature field, in that the basic catalytic mechanism is understood in the context of electronic changes leading to oxygen activation and the oxidation of a substrate (Scheme 1). Questions still exist about some of the electronics of Compound I and possibly other active species. More questions abound about the role of the protein structure in driving catalytic selectivity, including internal motion and conformational changes. Prediction of altered

catalytic activity due to small changes is problematic. Finally, there is a paucity of detailed information about interactions with accessory proteins.

Acknowledgments.

The author thanks Drs. F. K. Yoshimoto and M. J. Reddish for helpful comments and K. Trisler for assistance in preparation of the manuscript.

(Grant support). This work was supported in part by National Institutes of Health Grant R01 GM118122.

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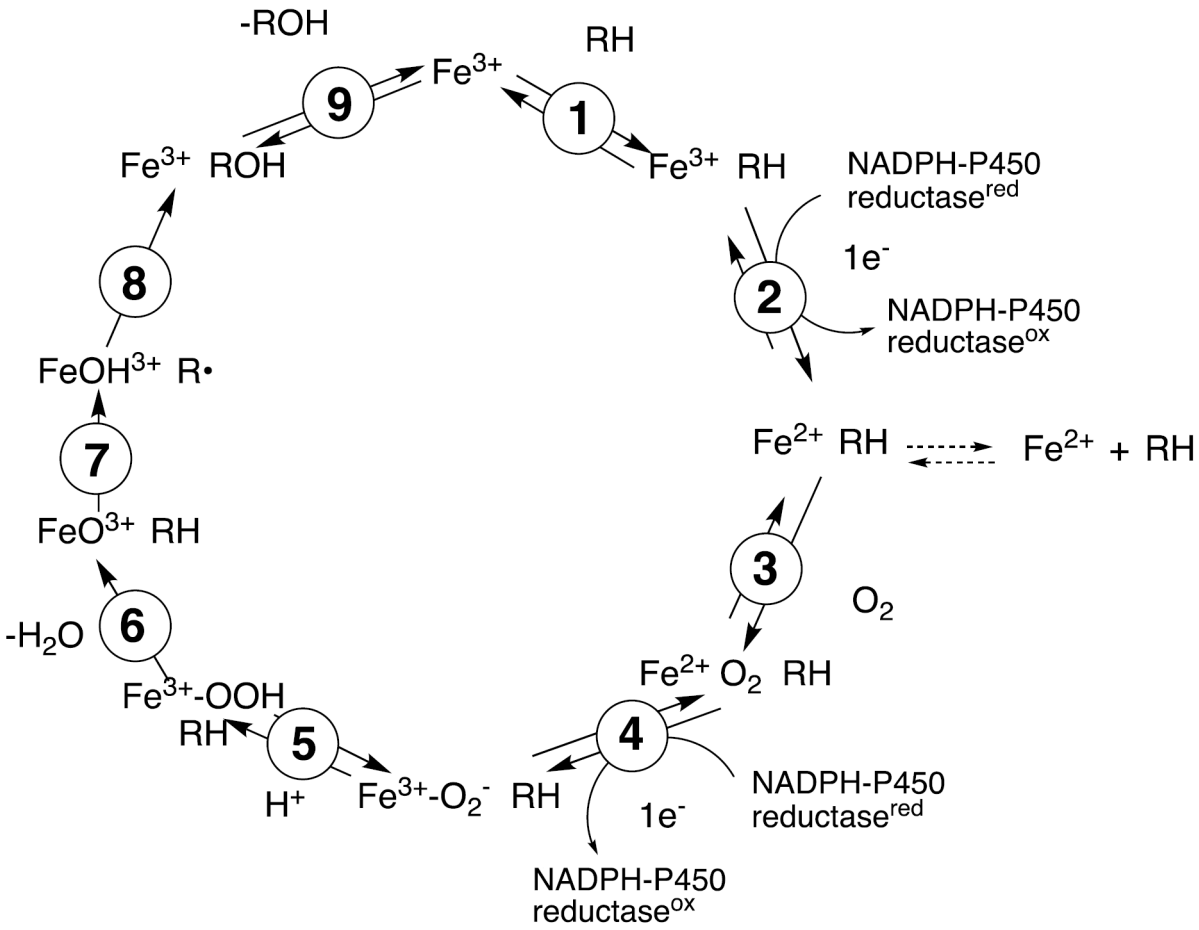
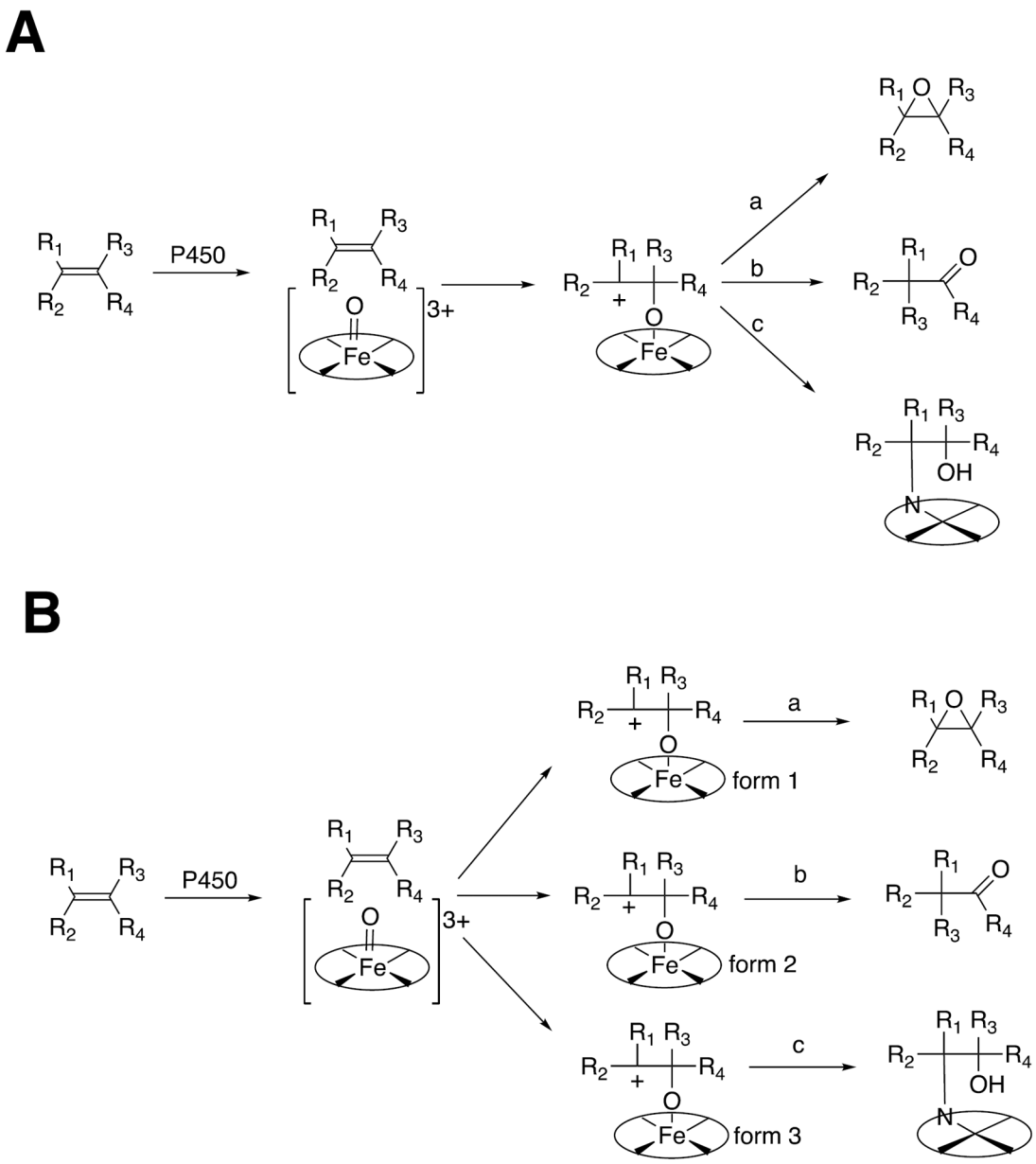


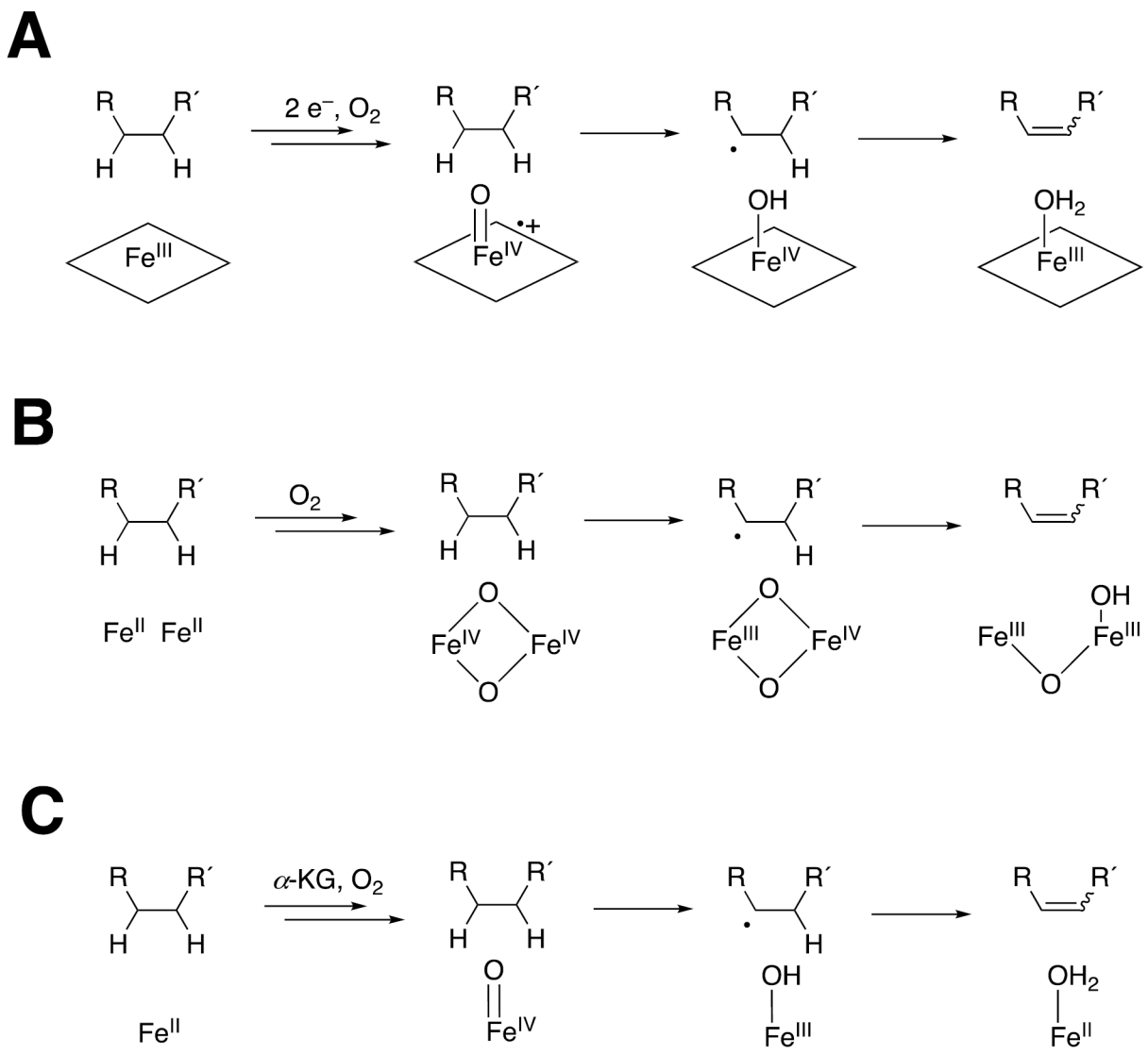
Scheme 1.

General catalytic cycle for P450 reactions.[6,19] In some cases cytochrome b_5 (b_5) can donate the electron in step **4**. b_5 can also donate the electron in step **2** although not as efficiently because of the thermodynamic barrier. In some P450s (some bacterial and mitochondrial P450s) steps **2** and **4** involve electron donation from ferredoxin proteins. The exact electronic distribution in the Fe-O entities between steps **4**–**8** is not well established in most cases.



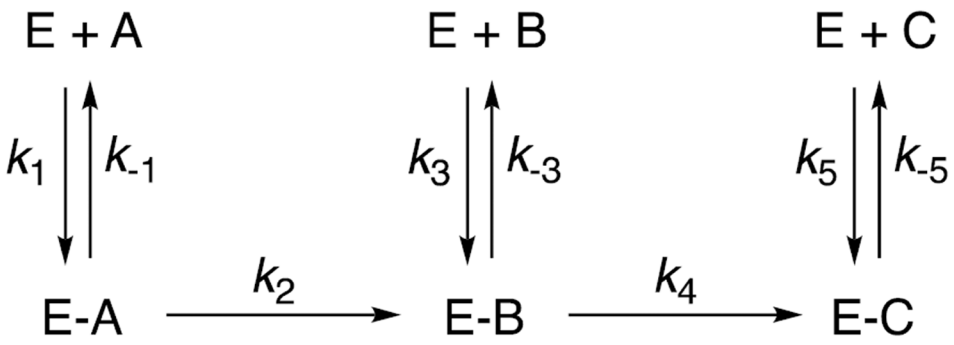
Scheme 2.

An example of the differences between explaining multiple reaction products by (A) a common intermediate, with variations due to the protein,[8,67] and (B)"two-state" (actually multistate) theory.[66].



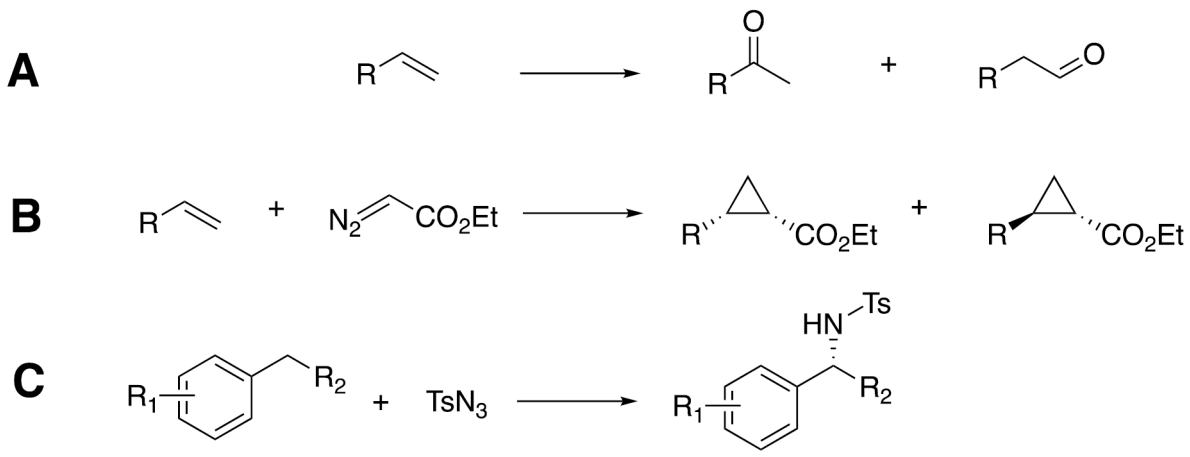
Scheme 3.

Potential desaturation mechanisms for oxygenases. (A) P450s; (B) non-heme diiron enzymes; (C) iron/α-ketoglutarate (α-KG) dioxygenases.[93]



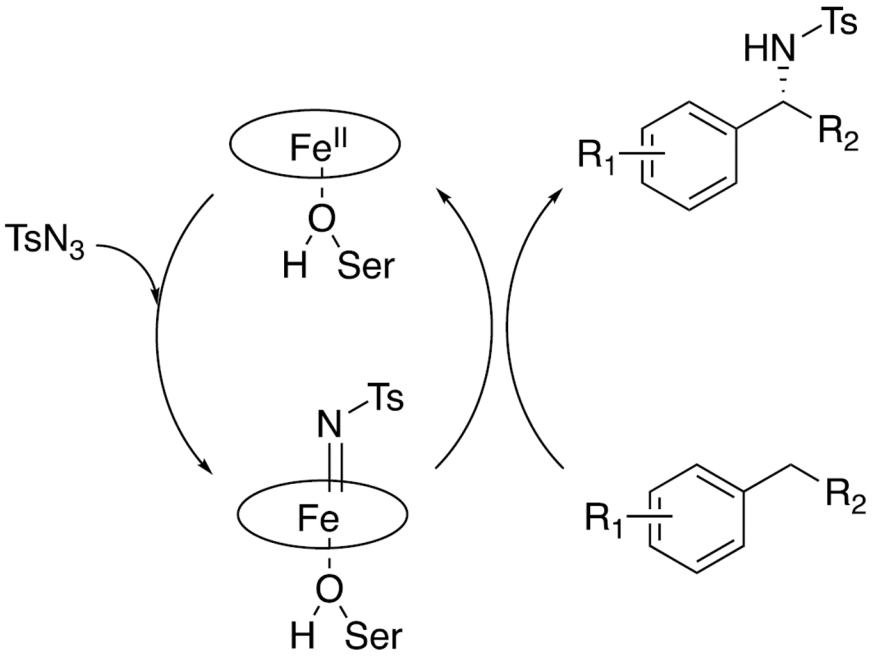
Scheme 4.

Processivity in multistep reactions. The enzyme (E) catalyzes the conversion of A → B → C, e.g, P450 17A1.[97] The degree of processivity is dominated by the ratio k_4 k_{-3}, although other rate constants can contribute.[104]

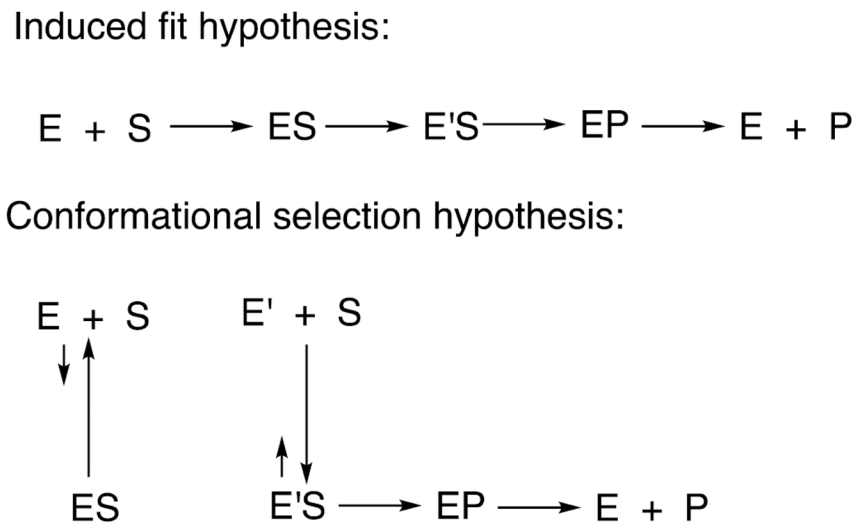


Scheme 5.

Some unusual reactions catalyzed by modified P450 catalysts.[135,144,145] Ts: tosyl.



Scheme 6.
A proposed mechanism of nitrene transfer for P450s.[144]



Scheme 7.

Induced fit vs. conformational selection hypotheses to explain multiplicity of enzyme conformations involved in catalysis.