0026-895X/97/010147-05$3.00/0

MOLECULAR PHARMACOLOGY, **51:**147–151 (1997).

Evidence for a Role of a Perferryl-Oxygen Complex, FeO^{3+}, in the *N*-Oxygenation of Amines by Cytochrome P450 Enzymes

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Received July 22, 1996; Accepted October 15, 1996

SUMMARY

Most cytochrome P450 (P450)-catalyzed reactions are believed to involve an FeO^{3+} intermediate as the actual oxygenating species. However, studies on the mechanism of steroid aromatization and subsequent model work have provided evidence that a peroxo-iron form (formally FeO_2^+) can be involved directly in some oxidations. The possible involvement of peroxo-iron was considered in P450-catalyzed *N*-oxygenations, because there is precedent for the use of H_2O_2 and organic peroxides in such reactions in the literature concerning synthetic and flavin reactions. The approach used was to compare P450 reactions involving the normal NADPH/NADPH-P450 reductase/O_2 system with those supported by the oxygen surrogates H_2O_2 (which can directly form FeO_2^+ and subsequently FeO^{3+}) and iodosylbenzene (which can form FeO^{3+} but not FeO_2^+). Iodosylbenzene was effective in supporting rabbit P450 1A2-catalyzed *N*,*N*-dimethyl-2-aminofluorene *N*-oxygenation, human P450 3A4-catalyzed quinidine *N*-oxygenation, rat P450 2B1-catalyzed oxidation of *N*-benzyl-(1-phenyl) cyclobutylamine to the *N*-hydroxyamine and nitrone, and rat P450 2B1-catalyzed and rabbit P450 2B4-catalyzed *N*-oxygenation of *N*,*N*-dimethylaniline (also *N*-demethylation). H_2O_2 also supported most of these reactions. A mutant of P450 2B4 with the substitution of alanine for threonine at position 302 has been shown to have decreased ability to catalyze reactions involving the putative FeO^{3+} but, presumably because of decreased ability to protonate the FeO_2^+ complex, to have enhanced activity in oxidative deformylation reactions believed to involve FeO_2^+. This mutant showed both decreased *N*,*N*-dimethylaniline *N*-demethylation and *N*-oxygenation activity. Although some contribution of an FeO_2^+ species to these reactions cannot be ruled out, formation of product in the iodosylbenzene-supported systems cannot be readily explained by an obligatory FeO_2^+ mechanism and the involvement of FeO^{3+} is concluded to be more likely.

P450 enzymes play important roles in the oxidation of many drugs and other xenobiotic chemicals as well as many compounds normally found in the body (1). (P450 is also termed "heme thiolate protein" by the Enzyme Commission; see Ref. 2 for nomenclature.) Some of the broad catalytic specificity can be attributed to the presence of a large number of individual proteins in this group, because more than 40 genes have been found in humans (2). However, it is also now apparent that a single P450 can catalyze most of the types of reactions found in the set. The current view is that the chemistry used by the different P450s is relatively uniform, with some exceptions, and that catalytic selectivity is predominantly a function of how the proteins bind potential substrates and position atoms toward the reactive center provided by the heme prosthetic group (1, 3).

One type of P450 reaction is heteroatom oxygenation, which has been identified in the transfer of oxygen to the atoms nitrogen, sulfur, phosphorus, and possibly iodine (4–8). Such transfers seem precluded from many peroxidases because of steric crowding (9, 10). *N*-Oxygenation, the subject of this report, had been recognized as being catalyzed by the flavoprotein termed "microsomal flavin-containing monooxygenase" (11) and was not considered to happen often in P450-catalyzed reactions. There is a mechanistic rationale for this (4). If amine oxidations proceed via single-electron oxidation to form aminium radicals, then dealkylation should dominate over oxygen "rebound" to the nitrogen due to facile deprotonation of α-hydrogens (4, 10). In some cases, α-deprotonation is not possible and *N*-oxygenation is prominent. For instance, with aryl and heterocyclic (primary) amines, there are no α-hydrogens. Quinidine cannot lose protons, because of Bredt's Rule, and forms an *N*-oxide (12). The conversion of azo compounds to azoxy can be rationalized in terms of nitrogen radical stability enhancement by the neighboring nitrogen lone pair (4). More recently, several examples of P450-catalyzed *N*-oxygenation have been identified in which

This work was supported in part by United States Public Health Service Grants R35-CA44353 and P30-ES00267 (to F.P.G.) and DK10339 (to M.J.C.).

ABBREVIATIONS: P450, cytochrome P450; HPLC, high performance liquid chromatography; Fp, NADPH-P450 reductase; PhIO, iodosylbenzene; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

α-hydrogens are available but *N*-oxides are formed (13–16). In all cases, however, *N*-oxygenation is accompanied by extensive *N*-dealkylation or products that can be rationalized by *N*-dealkylation. The mechanism basis of *N*-oxygenation is, therefore, still considered to involve aminium radical formation and partial oxygen rebound, with the partitioning to *N*-dealkylation being influenced by undescribed aspects of protein structure (10).

Although most of the P450 reactions are rationalized in terms of common chemistry involving the perferryl iron-oxygen complex (FeO^{3+}) (Fig. 1), evidence for the involvement of an alternative species has been obtained. The first two steps of oxidative removal of C-methyl groups in the metabolism of several steroids can be rationalized in terms of FeO^{3+} chemistry, but the third step involves the oxidative removal of a formyl group as HCO_2H. The proposition that the FeO_2^+ entity (Fe—OO^- in Fig. 1) is involved in such a reaction was first proposed by Akhtar (17). This proposal has been strengthened by model studies from Cole and Robinson (18) and by examples of such oxidative deformylation of monocyclic and bicyclic model compounds by nonsteroidogenic P450s (19–21).

The possible contribution of a FeO_2^+ (peroxy-iron) intermediate to the formation of *N*-oxides and hydroxyamines also can be considered. *N*-Oxides are prepared synthetically using H_2O_2 and peracids (22, 23). Furthermore, the mechanism of formation of *N*-oxides by microsomal flavin-containing monooxygenase and biomimetic models involves a functionalized hydroperoxide (24). However, these systems all yield only *N*-oxides, without *N*-dealkylation.

Details of these two possible mechanisms, sequential electron transfer followed by oxygen rebound and direct oxygenation by a ferric peroxide, are shown in Fig. 2, A and B, respectively. The possibility that the iron-peroxy complex is involved in *N*-oxygenation was raised in a previous report from this laboratory (16), but the hypothesis was not subjected to examination. Although direct proof for the role of an FeO_2^+ is unavailable in all cases, several indirect approaches have been applied to test the possibility, including the use of the oxygen surrogates H_2O_2 (potentially capable of forming $FeO_2^+ = Fe^{3+} + H_2O_2 - 2H^+$) and PhIO (which can form FeO^{3+} but not FeO_2^+) (19, 20) (Fig. 1). Another approach has been the use of a P450 2B4 T302A mutant, which seems to have attenuated ability to cleave FeO_2^+ and enhanced proficiency to catalyze oxidative deformylation reactions (21). Both approaches were used in the work on the mechanism of P450 *N*-oxygenation reported here.

Materials and Methods

Chemicals. [*methyl*-^{14}C]*N*,*N*-Dimethylaniline (10.1 mCi/mmol) was purchased from Sigma Chemical (St. Louis, MO) and purified by preparative HPLC before use (16). *N*,*N*-Dimethyl-2-aminofluorene (16) and *N*-benzyl(1-phenyl)cyclobutylamine (15) were prepared as described previously. The preparations of the respective oxides are described in the same references. Quinidine *N*-oxide was prepared as described previously (12): m.p. 145–152°, lit m.p. 148–150° (12); fast atom bombardment mass spectrometry, *m/z* 341 (MH^+, 100% relative abundance); UV (H_2O) λ_{max} 235, 280, 322, 334 nm. PhIO was prepared by hydrolysis of the diacetate (25). H_2O_2 (30% solution) was purchased from Fisher Scientific (Pittsburgh, PA).

Enzymes. P450 2B1 was purified from livers of phenobarbital-treated rats as described elsewhere (26). Modified constructs of human P450 1A2 (27) and 3A4 (28) were expressed in *Escherichia coli* and purified as described previously. A modified construct of rabbit P450 2B4, devoid of normal residues 2–27, and the T302A mutant (change of threonine to alanine at position 302) were expressed in *E. coli* as glutathione transferase proteins, purified by glutathione affinity chromatography, cleaved with thrombin, and purified as described previously (21, 29). Fp (30) and cytochrome b_5 (31) were purified from livers of phenobarbital-treated rabbits essentially as described.

Assays. Unless indicated otherwise in the tables (i.e., P450 3A4), all assays were performed in 0.10 M potassium phosphate buffer, pH 7.7, which contained 30 μM L-α-dilauroyl-*sn*-glycero-3-phosphocholine. The NADPH-dependent systems included the indicated amount of Fp plus an NADPH-generating system consisting of (final concentrations of) 0.5 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1 IU of yeast glucose 6-phosphate dehydrogenase/ml, plus catalase (0.18 μM) to quench any generated H_2O_2 (32). NADPH-dependent incubations were performed at 37° for 15 min. The H_2O_2-dependent incubations included 2.0 mM H_2O_2 (20) and were performed at 37° for 10 min. The PhIO-dependent incubations were performed at 23° for 30 sec (20, 33). Reactions were quenched by the addition of 2 volumes of CH_2Cl_2 for extraction. Control incubations were performed using P450s with

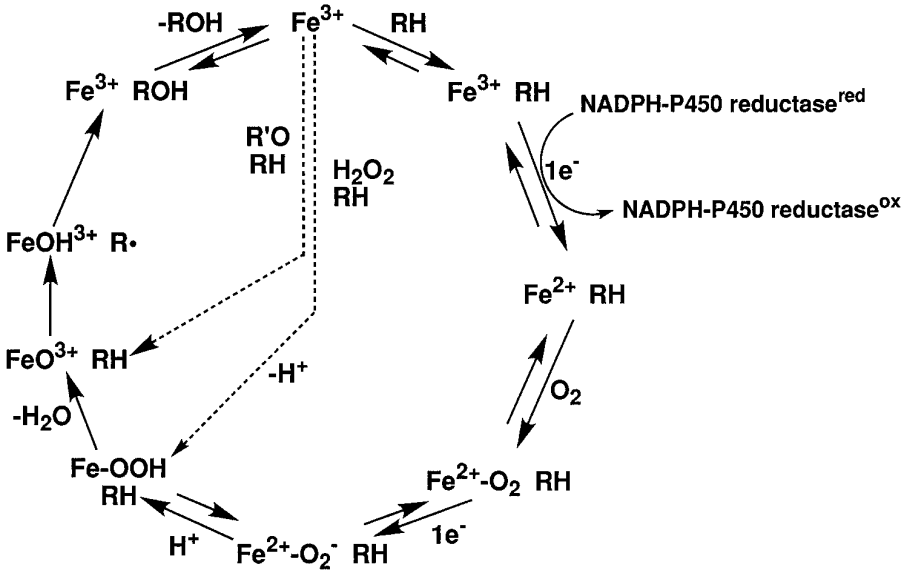

Fig. 1. General mechanism of P450 catalysis.

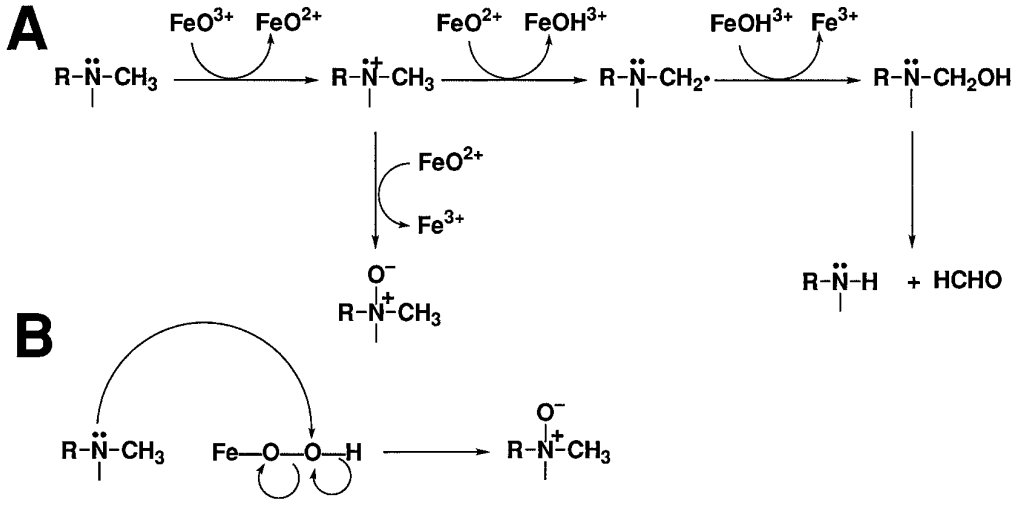

Fig. 2. Possible mechanisms of *N*-oxygenation by P450. A, Sequential electron transfer followed by oxygen rebound. B, direct oxygenation by a ferric peroxide.

cofactors (e.g., NADPH, H_2O_2, or PhIO) and with the cofactors in the absence of P450.

The analyses of products all were performed by HPLC as described previously, using detection by UV in the case of the *N*-benzyl(1-phenyl)cyclobutylamine products (15), fluorescence with quinidine *N*-oxide (12), and radioactivity in the case of the *N*,*N*-dimethylaniline products (16). The UV absorbance of 2-*N*,*N*-dimethylflourene *N*-oxide is appreciable compared with monocyclic analogs (16), and this compound was assayed directly by HPLC (A_{295}) rather than with the previously described isolation/reduction/HPLC method (16). External standards were used for HPLC. P450 concentrations were determined from Fe^{2+}·CO versus Fe^{2+} spectra using the method of Omura and Sato (34).

Results and Discussion

Rationale. The extent of product formation was compared in P450 systems supported by NADPH, H_2O_2, and PhIO to evaluate the role of the perferryl P450 intermediate, FeO^{3+} (Figs. 1 and 2). In previous studies with model aldehydes, it was concluded that reactions involved the FeO_2^{+} intermediate, instead of FeO^{3+}, if reactions were supported more effectively by H_2O_2 than NADPH and if PhIO was not able to support reactions (20). If the FeO^{3+} form is involved, H_2O_2 can still support the reaction. However, FeO_2^{+}-dependent reactions should not be supported by PhIO. This general approach was applied to four known *N*-oxygenation reactions catalyzed by four different P450s.

The choice of concentrations of cofactors (i.e., H_2O_2, PhIO) was based on literature precedents (20, 33). The oxygen surrogates are destructive to P450; PhIO is more so than H_2O_2 (32, 33, 35). The choices of time and concentration are based on the literature and preliminary trials with P450 2B1 dependent *N*,*N*-dimethylaniline *N*-oxygenation. On the basis of the previous work in this area (20), the times and cofactor concentrations should favor greater linearity of product formation in the H_2O_2-dependent system compared with the PhIO-dependent system. However, time course experiments were not performed in all cases; thus, the results are expressed as total products rather than rates.

***N*,*N*-Dimethyl-2-aminofluorene *N*-Oxygenation.** This reaction is catalyzed by P450 2B1, with an appreciable ratio of *N*-oxygenation/*N*-demethylation (16). NADPH-dependent *N*-oxygenation was also demonstrated with rabbit P450 1A2 (Table 1). The reaction was also supported by PhIO; the level of *N*-oxide formation seen with H_2O_2 was near the limit of detection.

Quinidine *N*-Oxygenation. Quinidine is oxidized in human liver microsomes, primarily by P450 3A4, to the 3′-hydroxy and *N*-oxide products (12). The amount of *N*-oxide formed with PhIO was as high as in the case of NADPH (Table 2). However, the amount formed in the presence of H_2O_2 was considerably higher than either the NADPH- or PhIO-based reaction.

***N*-Benzyl(1-phenyl)cyclobutylamine Oxidation.** This substrate had been used previously in studies involving mechanism-based inactivation of P450 2B1 and ring expansion, invoking an aminium radical intermediate (15). Surprisingly, the substrate was converted to a stable nitrone as one of the major products, apparently via an *N*-hydroxyamine. The nitrone could be formed from the *N*-hydroxyamine by oxygenation on nitrogen or alternatively by another mechanism involving initial oxidation of the nitrogen (1-electron oxidation) and abstraction of the α-hydrogen (with or without hydroxylation at the α-hydrogen).

Both of these products, the *N*-hydroxyamine and the nitrone, were seen in all three systems (NADPH, H_2O_2, PhIO) (Table 3), with the *N*-hydroxyamine favored in the case of NADPH and the nitrone in the case of H_2O_2.

***N*,*N*-Dimethylaniline *N*-Oxygenation.** This substrate was selected because of historic interest in the reaction (36, 37) and the opportunity to examine partitioning between

TABLE 1

***N*-Oxygenation of *N*,*N*-dimethyl-2-aminofluorene by rabbit P450 1A2**

The experiments were done with 5.3 nmol of rabbit P450 1A2 and, in the NADPH system, 7.5 nmol of Fp in a final volume of 1.0 ml. The concentration of *N*,*N*-dimethyl-2-aminofluorene was 50 μM. Values are means of triplicate experiments ± standard deviation.

System	*N*-Oxide formed
	nmol
NADPH	1.6 ± 0.6
H_2O_2	0.08 ± 0.08
PhIO	0.29 ± 0.06

TABLE 2

_N_-Oxygenation of quinidine by human P450 3A4

Experiments were performed with 0.65 nmol of P450 3A4 and, in the case of NADPH plus Fp incubations, 1.3 nmol of Fp, and 0.65 nmol of cytochrome b_5. All incubates included 0.50 ml of 50 mM potassium HEPES buffer, pH 7.7, containing a mixture of a 1:1:1 (w/w/w) mixture of L-α-dilauroyl-*sn*-glycero-3-phosphocholine, L-α-dioleyl-*sn*-glycero-3-phosphocholine, and bovine brain phosphatidylserine (20 μg/ml), 0.50 mM sodium cholate, 30 mM $MgCl_2$, and 200 μM quinidine (28). Values are means of triplicate experiments ± standard deviation.

System	*N*-Oxide formed
	nmol
NADPH	0.33 ± 0.10
H_2O_2	2.14 ± 0.17
PhIO	0.32 ± 0.02

TABLE 3

Oxygenation of _N_-benzyl-(1-phenyl)cyclobutylamine by rat P450 2B1

Incubations included 2.2 nmol of rat P450 2B1 and, in the NADPH system, 3.0 nmol Fp. The final volume was 1.0 ml in all cases, and the substrate concentration was 1.0 mM. Values are mean of triplicate experiments ± standard deviation.

System	*N*-Hydroxyamine formed	Nitrone formed
	pmol	*nmol*
NADPH	48 ± 14	1.9 ± 0.1
H_2O_2	14 ± 2	3.0 ± 0.2
PhIO	13 ± 5	0.8 ± 0.2

N-oxygenation and *N*-oxygenation reactions at the same site (16). The amount of *N*-oxygenation is low but finite (16), and radioactivity assays provide the only sensitive approach to examining rates, except for the isolation/reduction/rechromatography approach used previously (10). P450 2B1 had been used previously in assays (16). A rather homologous rabbit protein, 2B4, was examined because of its expected catalytic similarity and because the T302A mutant was available and had been used in studies on the role of the FeO_2^+ in the decarboxylation of model aldehydes (21). Thr302, apparently analogous to Thr268 of P450 101 ($P450_{cam}$), seems to be involved in the heterolytic cleavage of the O—O bond (38). The mutation was shown to facilitate the reaction believed to involve an FeO_2^+ entity but to attenuate reactions in which cleavage to FeO^{3+} is required (21).

P450 2B1-catalyzed *N*-demethylation was supported by NADPH and by H_2O_2 or PhIO, although not so effectively (Table 4). The differences were not so great in the case of P450 2B4, primarily because the NADPH-dependent reaction was slower. The three *N*-demethylation reactions, supported by the different cofactors, were all slower with the T302A mutant.

TABLE 4

_N_-oxygenation and _N_-demethylation of _N,N_-dimethylaniline by rat P450 2B1 and rabbit P450 2B4 derivatives

The system contained either 1.0 nmol of P450 2B1 plus 1.6 nmol of Fp or 0.50 nmol of P450 2B4 (or P450 2B4 T302A) plus 0.80 nmol of Fp, in a volume of 0.50 ml (Fp added only in the NADPH system). Results are expressed as means of duplicate experiments (no standard deviation indicated) or triplicate experiments (± standard deviation).

System	Product Formed		
	2B1	2B4	2B4, T302A
	pmol/nmol P450		
N-Methylaniline			
NADPH, Fp	240	96 ± 21	48 ± 8
H_2O_2	21	47 ± 5	5 ± 1
PhIO	21	25 ± 1	8 ± 5
N-Oxide			
NADPH, Fp	110 ± 35	180 ± 15	26 ± 6
H_2O_2	130 ± 60	180 ± 60	3 ± 3
PhIO	130	140 ± 15	67 ± 42

N-oxygenation by P450s 2B1 and 2B4 occurred to similar extents in the reactions supported by different cofactors (Table 4). With the T302A mutant, all reactions were attenuated, particularly the H_2O_2-dependent reaction. The decreased catalytic activity seen in the reactions supported by the oxygen surrogates may be the result of decreased stability of the T302A mutant to these oxidants. This aspect of the T302A mutants has not been examined previously (21).

The conclusions regarding the work with *N,N*-dimethylaniline reactions are that the mutation of Thr302 seems to decrease both *N*-demethylation and *N*-oxygenation. In all cases, a substantial amount of PhIO-dependent *N*-oxygenation was observed.

Conclusions. The potential similarity of the oxygenation of amines by P450s to reactions that occur with peracids and hydroperoxides (22, 23) and the flavin hydroperoxide intermediate of the flavin-containing monooxygenase (11) suggested that P450 might use a recently discovered mechanism involving the FeO_2^+ entity. However, examination of four different *N*-oxygenations with four different P450s does not provide any evidence for such a view.

The results show that the PhIO-dependent system formed products, as did the H_2O_2-dependent system (Tables 1–4). It is also accepted that the FeO_2^+ species, formed by the addition of H_2O_2, breaks down to give the FeO^{3+} species. The results obtained from these experiments, which are designed to distinguish between FeO_2^+ and FeO^{3+}, seem more consistent with the key role of FeO^{3+} as the active species accountable for product formation in this system rather than FeO^{2+}. Further, mutation of P450 2B4 Thr302 to alanine attenuated the NADPH-dependent *N*-oxygenation and *N*-dealkylation of *N,N*-dimethylaniline, in contrast to what would have been expected if the FeO_2^+ entity had a dominant role in oxygenation. These results can be rationalized by the limited ability of a nucleophilic intermediate ($FeO_2^+ = Fe^{2+}$—O—O^-) to attack a nucleophilic site, whether or not it is protonated (Fig. 2B).

An *N*-oxygenation mechanism is preferred that involves 1-electron oxidation of the nitrogen atom as a first step and subsequent oxygen rebound (10, 39), as proposed for P450 sulfur oxygenations (6, 40). Details of the reaction remain unknown, particularly whether a FeO_2^+/aminium radical pair undergoes additional electron transfer before rebound (10). However, the conclusion is reached here that the FeO^{3+} entity is an inherent part of the mechanism of *N*-oxygenation by many P450s.

Acknowledgments

We thank C. G. Turvy for purifying Fp, M. V. Martin for purifying P450s 2B1 and 3A4, Prof. P. Anzenbacher for preparing P450 1A2, Dr. Y. Seto for synthesizing *N,N*-dimethyl-2-aminofluorene and its *N*-oxide, L. A. Cowart for performing some of the preliminary experiments, Prof. T. L. Macdonald (University of Virginia, Charlottesville, VA; all others from Vanderbilt University, Nashville, TN) for his comments on the manuscript, and E. Rochelle for assistance in preparation of the manuscript.

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