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# Peroxo-iron and oxenoid-iron species as alternative oxygenating agents in cytochrome P450-catalyzed reactions: Switching by Threonine-302 to Alanine mutagenesis of cytochrome P450 2B4

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**ABSTRACT** **Among biological catalysts, cytochrome P450 is unmatched in its multiplicity of isoforms, inducers, substrates, and types of chemical reactions catalyzed. In the present study, evidence is given that this versatility extends to the nature of the active oxidant. Although mechanistic evidence from several laboratories points to a hypervalent iron-oxenoid species in P450-catalyzed oxygenation reactions, Akhtar and colleagues [Akhtar, M., Calder, M. R., Corina, D. L. & Wright, J. N. (1982) *Biochem. J.* 201, 569–580] proposed that in steroid deformylation effected by P450 aromatase an iron-peroxo species is involved. We have shown more recently that purified liver microsomal P450 cytochromes, including phenobarbital-induced P450 2B4, catalyze the analogous deformylation of a series of xenobiotic aldehydes with olefin formation. The investigation presented here on the effect of site-directed mutagenesis of threonine-302 to alanine on the activities of recombinant P450 2B4 with N-terminal amino acids 2–27 deleted [2B4 (Δ2–27)] makes use of evidence from other laboratories that the corresponding mutation in bacterial P450s interferes with the activation of dioxygen to the oxenoid species by blocking proton delivery to the active site. The rates of NADPH oxidation, hydrogen peroxide production, and product formation from four substrates, including formaldehyde from benzphetamine N-demethylation, acetophenone from 1-phenylethanol oxidation, cyclohexanol from cyclohexane hydroxylation, and cyclohexene from cyclohexane carboxaldehyde deformylation, were determined with P450s 2B4, 2B4 (Δ2–27), and 2B4 (Δ2–27) T302A. Replacement of the threonine residue in the truncated cytochrome gave a 1.6- to 2.5-fold increase in peroxide formation in the presence of a substrate, but resulted in decreased product formation from benzphetamine (9-fold), cyclohexane (4-fold), and 1-phenylethanol (2-fold). In sharp contrast, the deformylation of cyclohexane carboxaldehyde by the T302A mutant was increased about 10-fold. On the basis of these findings and our previous evidence that aldehyde deformylation is supported by added $H_2O_2$, but not by artificial oxidants, we conclude that the iron-peroxy species is the direct oxygen donor. It remains to be established which of the many other oxidative reactions involving P450 utilize this species and the extent to which peroxo-iron and oxenoid-iron function as alternative oxygenating agents with the numerous isoforms of this versatile catalyst.**

The cytochrome P450 (P450) gene superfamily includes numerous hemoproteins involved in the metabolism of steroid hormones, vitamins, retinoids, eicosanoids, and other physiologically occurring compounds, as well as of an almost unlimited variety of drugs, pesticides, carcinogens, and other xenobiotics (1). These versatile catalysts are thought to use as an active oxidant a hypervalent iron-oxenoid species comparable to compound I of peroxidases (2–5). Although the identity of this oxidant in P450-catalyzed reactions has not been rigorously established, mechanistic evidence points to the involvement of such a species in hydroxylation reactions and other transformations catalyzed by these enzymes (5, 6). However, in examining the terminal step in steroid demethylation catalyzed by P450 aromatase, Akhtar and colleagues (7) observed that the pattern of isotopic label distribution and the incorporation of an oxygen atom from molecular oxygen into the products was inconsistent with an oxenoid oxidant. Furthermore, they hypothesized that the active oxidant in the aromatase-catalyzed carbon—carbon bond cleavage step and related reactions effected by lanosterol 14α-demethylase, progesterone 17,20-lyase, and pregnenolone 17,20-lyase-$\Delta^{16}$-desaturase was a nucleophilic, iron-peroxo species (7, 8). Subsequently, other investigators (9–12) proposed a similar intermediate, and our laboratory (13, 14) showed that purified isoforms of liver microsomal P450 catalyze the oxidative deformylation of a variety of xenobiotic aldehydes to olefins and formate, a reaction comparable in functional group transformation to the terminal carbon—carbon bond cleavage step catalyzed by aromatase and lanosterol 14α-demethylase. Consistent with an iron-peroxo species as the active oxidant in this reaction was the demonstration that of several agents known to support many P450-catalyzed reactions in the absence of $O_2$ and NADPH, $H_2O_2$ was capable of supporting olefin formation in the oxidative deformylation of cyclohexane carboxaldehyde, but iodosobenzene, *m*-chloroperbenzoic acid, and cumyl hydroperoxide were not (14). More recently, a bicyclic steroid analog, 3-oxodecalin-4-ene-10-carboxaldehyde, which corresponds to the A and B rings of the 19-oxo androgen intermediate in the aromatase reaction, has been shown to be oxidized to formate and the expected aromatic product, 3-hydroxytetrahydronaphthalene, with the same stereochemical specificity as in the terminal step of the aromatase-catalyzed reaction (15, 16). The iron-peroxo concept has recently been extended to the nitric oxide-generating step in the reaction of nitric oxide synthase (17). It may be noted that the iron-peroxo and iron-oxenoid species are fundamentally distinct oxidants, the former being nucleophilic and the latter electrophilic in character.

In this paper we have used site-specific mutagenesis of P450 2B4 (rabbit liver) (2B4) to determine amino acid residues at the active site that may control the relative extent of the oxenoid and peroxo pathways. The extensive crystallographic and mutagenic studies on bacterial $P450_{cam}$ and sequence homology alignments with numerous mammalian P450s served to identify threonine-302 of P450 2B4 as a candidate for

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*Abbreviations*: P450, cytochrome P450; 2B4, P450 2B4 (rabbit liver); 2B4 (Δ2–27), recombinant P450 2B4 with N-terminal amino acids 2–27 deleted; 2B4 (Δ2–27) T302A, truncated P450 2B4 with threonine-302 mutated to alanine.

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mutagenesis in this study. Raag *et al.* (18) have evaluated an earlier proposal on hydrogen bonding (19) and have discussed oxygen activation in light of two potential pathways identified for proton delivery to the active site via an internal solvent channel between threonine-252 and glutamate-366 or through a hydrogen bonding network extending from the surface of the protein to the heme environment. Although a crystal structure of oxygen bound to $P450_{cam}$ has not been determined, molecular modeling places threonine-252 in bonding distance to the putative heme dioxygen complex, and this residue has been postulated to play a role in the activation of dioxygen to the oxenoid species by serving as a link for proton delivery to the active site (18, 19). In support of such a role for threonine-252 in the catalytic cycle of $P450_{cam}$, its mutation to valine or alanine resulted in a protein essentially devoid of camphor hydroxylation activity, whereas neither substrate binding nor the rate of the first electron transfer to the heme center was affected. The uptake of reducing equivalents from NADH was largely uncoupled from substrate hydroxylation and resulted in the nonproductive formation of $H_2O_2$ or $H_2O$ as shown by Martinis *et al.* (20) and Imai *et al.* (21). More recently, Kimata *et al.* (22) have replaced T252 in $P450_{cam}$ by the unnatural amino acid *O*-methylthreonine. The mutation resulted in a protein that was identical to the wild-type enzyme in its catalytic properties, suggesting that T252 does not function directly as a proton donor. The possibility remains, however, that the altered residue had undergone O-demethylation. From sequence homology alignments a highly conserved threonine corresponding to that in position 252 of $P450_{cam}$ has been identified in most known P450 sequences, an exception being $P450_{EryF}$, which has a valine instead (23–26). The conserved threonine of several mammalian P450 isoforms has been mutated to examine its role in catalysis. For example, replacement of threonine-301 by histidine or valine in rabbit liver laurate ($\omega-1$) hydroxylase and testosterone 16$\alpha$-hydroxylase P450s caused a significant loss of hydroxylation activity by these enzymes (27, 28), and mutagenesis of threonine-319 of P450 1A2 resulted in loss of activity in benzphetamine demethylation but not in 7-ethoxycoumarin O-deethylation (29, 30). Recently the mutation of threonine-268 of $P450_{BM-3}$ has been shown to result in no significant alteration of the heme domain structure (31). However, the rates of NADPH oxidation and oxygen consumption are decreased, and a significant loss in laurate hydroxylation activity with uncoupling to produce $H_2O_2$ and $H_2O$ has been noted. The heme domain of this P450 has significantly more sequence homology than $P450_{cam}$ with respect to the microsomal P450s (32).

Because the oxidative deformylation of various xenobiotic aldehydes to yield olefins and formate is believed to involve the iron-peroxo intermediate as the active oxidant, we postulated that disruption of the pathway leading from the iron-peroxo to the iron-oxene species should result in loss of oxenoid-dependent hydroxylation chemistry and enhancement of iron-peroxo-dependent reactions. We have briefly reported our findings on this problem (33, 34), and herein we present our studies involving the mutagenesis of the conserved threonine (T302) to alanine in the P450 2B4 sequence. The results indicate that the hydroxylation of several substrates is greatly diminished by this mutation, but that the deformylation-desaturation reaction is strikingly enhanced.

## MATERIALS AND METHODS

**Materials.** Electrophoretically homogeneous P450 2B4 (specific content, 15.4 nmol per mg of protein) and NADPH-cytochrome P450 reductase (specific activity, 30.2 units per mg at 30°C) were obtained from liver microsomes of male New Zealand rabbits that had been treated with phenobarbital, as described (35, 36). Analytical grade reagents were obtained commercially and used as such unless otherwise indicated.

**Cloning and Heterologous Expression of $NH_2$-Terminal-Truncated P450s.** Shortened P450 2B4 and the truncated P450 2B4 with threonine-302 mutated to alanine (2B4 (Δ2–27) T302A), both lacking residues 2–27, were expressed in *Escherichia coli* as fusion proteins with glutathione *S*-transferase linked to the $NH_2$ terminus of the cytochrome. The vectors used were constructed from the Pharmacia vector pGEX-KN modified to include a kinker region encoding a sequence that improves the efficiency of thrombin cleavage of the fusion protein. The 2B4 (Δ2–27) clone was constructed as described by Pernecky *et al.* (37). Threonine-302 was changed by the PCR method to alanine; the 5′ amplification primer (GC AAG CTT GCC GGC ACC GAG GCC ACC AGC ACC ACC C) extending from positions 892 to 919 in the cDNA sequence of 2B4 (38) contained an A to G substitution at position 904 to produce the desired mutation and an *Nae*I site for cloning into the vector. The first eight nucleotides (GCAAGCTT), which are not in the 2B4 gene sequence, were present to improve the cutting efficiency of *Nae*I. The PCR product was digested with *Nae*I and *Rsr*II and cloned into the similarly digested shortened 2B4 construct to obtain 2B4 (Δ2–27) T302A. The DNA sequence of this region was determined to ensure fidelity of the *Taq* DNA polymerase during the reaction. The proteins were expressed in *E. coli* MV1304 cells as described (37). Typical yields of the recombinant proteins were from 50 to 300 nmol of P450 per liter of culture. For purification of the fusion proteins, the harvested cells were suspended in 30 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl, 20% glycerol, and 0.1% 2-mercaptoethanol (PBS), and then treated with *n*-octyl β-D-glucopyranoside (1% final concentration) with stirring for 20 min and lysed by two passes in a French pressure cell. All procedures were carried out at room temperature unless otherwise stated. The lysate was diluted with 1 vol of PBS and centrifuged at 140,000 × *g* to remove insoluble components. The clear supernatant fraction was applied to a glutathione-agarose column (40 nmol of P450 per ml of bed volume) that had been equilibrated with PBS containing 0.3% octyl glucoside (PBSO). The matrix was washed with 10 column vol of 100 mM Tris·Cl buffer, pH 7.4, containing 120 mM NaCl, 20% glycerol, and 0.3% octyl glucoside (TBSO). The contents of the column were then extruded, and the fusion protein was eluted from the matrix by stirring at 4°C overnight with an equal volume of TBSO buffer containing 20 mM glutathione and then several times for about 1 h with an equal volume of the same buffer. The pooled eluate was dialyzed against Tris-buffered saline (TBS) to remove glutathione and the detergent, and the fusion protein was treated at room temperature for 36 h with 8 units of thrombin per nmol of P450. The mixture was then passed over a glutathione–agarose column to remove glutathione *S*-tranferase and any remaining uncleaved fusion protein. The purified material was concentrated by dialysis overnight at 4°C against 2 liters of 100 mM potassium phosphate (pH 7.4), containing 20% glycerol and 0.1 mM EDTA, in a ProDicon concentrator (Spectrum Laboratories, Houston) to yield preparations that had a specific content of from 7.2 to 11.2 nmol of P450 per mg of protein.

**Enzymatic Reactions and Analytical Procedures.** A typical reaction mixture contained 0.1 nmol of P450 2B4, 2B4 (Δ2–27), or 2B4 (Δ2–27) T302A (except that when aldehyde deformylation was to be determined the amount of P450 was 0.2 nmol and 100 units of catalase and 50 units of superoxide dismutase were included), 0.2 nmol of reductase, 30 μg of dilauroylglycerol-3-phosphocholine, 100 μmol of potassium phosphate buffer (pH 7.4), substrate (1.0 μmol of benzphetamine, cyclohexane, or cyclohexane carboxaldehyde, or 10 μmol of 1-phenylethanol) in a final volume of 1.0 ml. All reactions were initiated by the addition of 0.15 μmol of NADPH and carried out at 30°C, except that the conversion of cyclohexane carboxaldehyde to cyclohexene was determined at 37°C. Formaldehyde formed from benzphetamine was mea-

Table 1. Rates of NADPH oxidation and hydrogen peroxide formation by P450 2B4 and variants in the reconstituted enzyme system

| Substrate | NADPH oxidation, nmol/min per nmol of P450 | | | $H_2O_2$ formation, nmol/min per nmol of P450 | | |
|---|---|---|---|---|---|---|
| | 2B4 | 2B4 (Δ2–27) | 2B4 (Δ2–27) T302A | 2B4 | 2B4 (Δ2–27) | 2B4 (Δ2–27) T302A |
| None | 16 ± 1 | 10 ± 1 | 16 ± 1 | 9 ± 1 | 9 ± 1 | 10 ± 1 |
| Benzphetamine | 121 ± 6 | 40 ± 1 | 42 ± 1 | 26 ± 3 | 13 ± 1 | 32 ± 3 |
| 1-Phenylethanol | 38 ± 2 | 18 ± 2 | 25 ± 1 | 16 ± 2 | 11 ± 1 | 22 ± 1 |
| Cyclohexane | 52 ± 2 | 24 ± 1 | 25 ± 1 | 19 ± 2 | 13 ± 1 | 21 ± 1 |
| Cyclohexane carboxaldehyde | 59 ± 2 | 21 ± 1 | 33 ± 1 | 50 ± 1 | 8 ± 1 | 18 ± 1 |

sured by the method of Cochin and Axelrod (39) and acetophenone formed from 1-phenylethanol was analyzed by reversed-phase HPLC as described by Vaz and Coon (40). Cyclohexanol formed from cyclohexane was analyzed by gas chromatography as follows: the reactions were quenched by the addition of 0.2 ml of 1 M NaOH, and 1.0 μmol of 1-hexanol was added as an internal standard. The reaction mixture was extracted with 2.0 ml of chloroform, the extract was concentrated to approximately 50 μl under reduced pressure in a vacuum centrifuge, and 1.0 μl of the residual solution was injected onto a 6-ft Supelco 60/80 Carbopack B column containing 4% Carbowax 20M and 0.8% KOH, operated under isothermal conditions at 160°C. The carrier gas was nitrogen at a flow rate of 22 ml/min. Under these conditions cyclohexanol was eluted at 8 min and the internal standard at 13 min. The integrated peak areas for cyclohexanol and 1-hexanol were linear with respect to concentration in the range from 10 nmol to 200 nmol and 100 nmol to 1000 nmol, respectively. Cyclohexene, the major product formed from cyclohexane carboxaldehyde, and cyclohexane, a minor product, were determined by gas chromatography of the head space of reaction mixtures that had been incubated in septum-sealed reaction vials. At the end of the incubation the vial was cooled in an ice-water bath and 0.1 ml of 30% perchloric acid was injected to quench the reaction. The vial was then heated at 60°C for 20 min and 1.0 ml of the head space gas was removed with a gas-tight syringe and injected onto a 6-ft Supelco 60/80 Carbopack B column containing 4% Carbowax 20M and 0.8% KOH. The carrier gas was helium at a flow rate of 27 ml/min. The column was maintained at 130°C for 7 min and the temperature was then raised to 210°C for 10 min. Cyclohexane and cyclohexene were eluted at 4.7 and 5.4 min, respectively. Standards were run in an identical manner; peak areas were linear over a concentration range from 0.2 to 2.0 nmol for both hydrocarbons. NADPH disappearance was measured by the decrease in absorbance at 340 nm. For the determination of $H_2O_2$, reactions were quenched with 0.2 ml of 45% trichloroacetic acid, followed by centrifugation to remove the precipitated material, and 1.0 ml of the supernatant solution was used for the determination of peroxide according to the method of Hildebrandt *et al.* (41). Under these conditions, NADPH disappearance, $H_2O_2$ formation, and the appearance of oxidation products from a series of substrates were all linear with time. In control experiments, P450 was omitted from the reaction mixtures or added after it had been heat-inactivated. Rate constants for initial electron transfer and binding constants for benzphetamine were determined according to published procedures (42, 43). The results presented in the tables are from an average of two or more experiments.

## RESULTS AND DISCUSSION

We chose to examine the effect of mutagenesis on truncated P450 2B4, which we are studying with respect to membrane targeting, rather than on the full-length protein. Use of the fusion protein with glutathione-*S*-transferase (37) along with the thrombin cleavage methodology allows for rapid purification of the expressed cytochrome so that about 100 nmol of purified P450 2B4 (Δ2–27) or the 2B4 (Δ2–27) T302A mutant are routinely obtained from a 1-liter culture of *E. coli* MV1304 cells. As noted in an earlier study, the truncated 2B4 retains the ability to function as a monooxygenase with all substrates examined, but the activities were found to be between 35 and 80% of those of the full-length protein in the various reactions (37). The reason for the somewhat lower activity of the truncated protein is at present unclear. A comparable $NH_2$-terminal truncation of P450 2E1 was shown previously to have no effect on the catalytic activity (44, 45). In the present study the mutation of threonine-302 to alanine resulted in no significant alteration in the ferric UV/visible spectrum or of the carbon monoxide difference spectrum of the 2B4 (Δ2–27) and the 2B4 (Δ2–27) T302A proteins relative to that of the purified liver microsomal "wild-type" 2B4 protein (data not shown).

The rates of NADPH oxidation and $H_2O_2$ formation were measured in the absence of a substrate and in the presence of benzphetamine, 1-phenylethanol, cyclohexane, or cyclohexane carboxaldehyde with the three forms of 2B4 in a reconstituted system, as shown in Table 1. Electron transfer, as judged by NADPH oxidation, was more rapid when any of the substrates was added to the system containing any of the three cytochromes. Particularly striking was the effect of benzphetamine, with an increase of more than 7-fold in the rate of NADPH consumption with the full-length enzyme. The truncated proteins generally catalyzed NADPH oxidation at a decreased rate as compared to intact 2B4, the only exception being the T302A mutant with no substrate present. With each protein the rate of peroxide formation was also generally increased with a substrate present, except for 1-phenylethanol and cyclohexane carboxaldehyde with P450 2B4 (Δ2–27). Trunca-

Table 2. Rates of substrate oxidation by P450 2B4 and variants in the reconstituted enzyme system

| Substrate | Product determined | Reaction rate, nmol/min per nmol of P450 | | |
|---|---|---|---|---|
| | | 2B4 | 2B4 (Δ2–27) | 2B4 (Δ2–27) T302A |
| Benzphetamine | Formaldehyde | 53.0 ± 2.0 | 27.0 ± 0.4 | 3.0 ± 0.2 |
| 1-Phenylethanol | Acetophenone | 18.4 ± 0.3 | 4.0 ± 0.0 | 2.5 ± 0.0 |
| Cyclohexane | Cyclohexanol | 20.0 ± 0.2 | 4.4 ± 0.7 | 1.0 ± 0.1 |
| Cyclohexane carboxaldehyde | Cyclohexene | 0.27 ± 0.0 | 0.15 ± 0.0 | 1.47 ± 0.1 |

Table 3. Initial rates of electron transfer and substrate binding constants for P450 cytochromes

| P450 | Substrate | Rate constant for P450 reduction ($k$), $min^{-1}$ | Binding constant ($K_s$), mM |
|---|---|---|---|
| 2B4 | None | 375 ± 15 | |
| | 1-Phenylethanol | 334 ± 25 | |
| | Benzphetamine | | 210 ± 20 |
| 2B4 (Δ2–27) | None | 363 ± 08 | |
| | 1-Phenylethanol | 353 ± 18 | |
| | Benzphetamine | | 135 ± 15 |
| 2B4 (Δ2–27) | None | 366 ± 12 | |
| T302A | 1-Phenylethanol | 339 ± 19 | |
| | Benzphetamine | | 150 ± 12 |

tion alone of 2B4 gave no increase in peroxide formation with or without a substrate present. Perhaps the most pertinent comparison from these results is the effect on the truncated enzyme of replacement of threonine-302 by alanine, which without substrate present resulted in a 1.6-fold increase in NADPH oxidation but no increase in peroxide production, and with substrates present gave variable effects on NADPH consumption but consistent increases in the yield of peroxide, ranging from 1.6- to 2.5-fold with the four substrates examined.

As indicated in Table 2, 2B4 (Δ2–27) is approximately 20 to 50% as active as the full-length enzyme in the N-demethylation of benzphetamine, the oxygenation of cyclohexane and 1-phenylethanol to give cyclohexanol and acetophenone, respectively, and the deformylation of cyclohexane carboxaldehyde to yield cyclohexene. This is consistent with previous results with several substrates, including *N,N*-dimethylaniline and *p*-nitrophenetole (37). The most important comparison, however, is of the reaction rates with the truncated cytochrome and the corresponding T302A mutant. The replacement of the threonine residue reduced the activities with benzphetamine, cyclohexane, and phenylethanol to one-ninth, one-fourth, and one-half, respectively. In this connection, generation of the iron-oxene oxidant is believed to require protonation of the iron-peroxy intermediate for cleavage of the O—O bond, as stated above, and additionally, Brinigar *et al.* (46) showed some years ago that heme-dioxygen complexes are unstable in protic solvents and are rapidly autoxidized, presumably due to protonation of the oxygen. Thus, solvent protons in the active site could strongly influence the course of reactions of the heme-peroxy intermediate. Raag *et al.* (18) have concluded from analysis of the structures of $P450_{cam}$ and its T252A variant that the greater availability of solvent protons in the latter protein is probably responsible for the uncoupling and that a highly orchestrated protonation of the iron-peroxy intermediate must occur at the active site to generate the iron-oxene species. A recent study by Miller and White (47) suggests that P450 2B4 retains water in the active site even in the presence of a hydrophobic substrate, which may account for the partial uncoupling observed with this enzyme. Alternatively, the solvent may serve as a protic source for cleavage of the O—O bond, partially substituting for threonine-302 in 2B4 (Δ2–27) T302A. This may account for the remaining slight activity in the benzphetamine and cyclohexane hydroxylation reactions catalyzed by the mutant enzyme. In the case of 1-phenylethanol oxidation to acetophenone, however, a reaction known to occur via hydroxylation of the benzylic carbon (40), as much as one-half of the activity is retained by our T302A mutant. It may be noted in this connection that $P450_{EryF}$, which is highly unusual in lacking the conserved threonine in the active site, is competent in the hydroxylation of the erythromycin precursor 6-deoxyerythronolide B (26). From such substrate structure-function relationships and analysis of the crystal structure of $P450_{EryF}$ (48), Poulos and colleagues (49) have suggested that a novel substrate-assisted acid catalysis is used in the oxygen activation step. Presumably, a substrate hydroxyl group in conjunction with ordered water in the active site may provide the necessary hydrogen bonding network between the active site and the bulk solvent such that a proton can be provided for cleavage of the iron-peroxo complex to generate the iron-oxene species. Thus, we propose that in a similar way the hydroxyl group of 1-phenylethanol in conjunction with water molecules in the active site may replace the proton delivery network disrupted by the T302A mutation in P450 2B4 (Δ2–27). It should also be noted that while the oxidation of 1-phenylethanol was demonstrated to proceed by a stepwise hydrogen atom abstraction and oxygen rebound mechanism, the oxidant was assumed to be an iron-oxenoid species (40). From our results it is apparent that threonine-302, while influencing the generation of the active oxidant for hydroxylation reactions, appears not to be uniquely responsible for promoting cleavage of the O—O bond of the iron-peroxo intermediate to the iron-oxenoid species.

In sharp contrast to the decreased oxidation of benzphetamine, cyclohexane, and 1-phenylethanol by the T302A mutant, the deformylation of cyclohexane carboxaldehyde to yield cyclohexene was increased about 10-fold over that of truncated 2B4 and more than 5-fold over that of intact 2B4 (Table 2). On the basis of this finding and that of previous studies with $H_2O_2$ and artificial oxidants (14), we conclude that the reaction

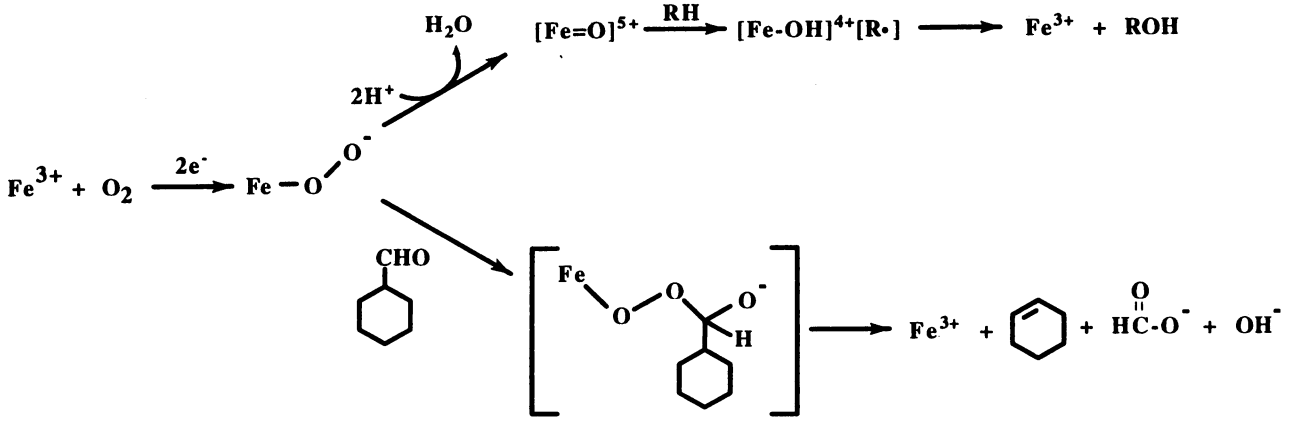


FIG. 1. Proposed scheme showing alternative routes for substrate oxidation by cytochrome P450, where Fe represents the heme iron atom at the active site, RH a substrate, and ROH the corresponding product in the oxenoid-supported pathway. Cyclohexane carboxaldehyde, a typical carbonyl substrate in the peroxide-supported pathway, is shown as producing a peroxyhemiacetal intermediate that yields formate with desaturation in the remaining structure.

involves peroxy-iron rather than oxenoid-iron as the active oxidant. It is important to note that the decreased oxidation of benzphetamine, 1-phenylethanol, and cyclohexane by the T302A mutant is not due to a decreased rate of reduction of ferric P450 or poor substrate binding, as indicated by the results presented in Table 3. In additional experiments not presented here, the E301L and T303A mutants of the truncated 2B4 P450 were expressed in *E. coli* and purified. The 301 mutant had little activity in all of the reactions examined and the 303 mutant was comparable to the truncated 2B4 in its activity. Of particular interest, neither of these variants displayed the higher activity in aldehyde deformylation reported here for the T302A protein (H. M. Peng, personal communication).

As shown in the accompanying scheme (Fig. 1), a critical difference between the two pathways is the requirement for protons at the active site for cleavage of the oxygen—oxygen bond in the oxenoid route that results in the hydroxylation of a substrate, RH, to form ROH, but not for the deformylation-desaturation reaction with an aldehyde when peroxide serves as the direct oxygen donor. Thus, when the usual route for protonation of the iron-peroxide intermediate in the active site is perturbed, as with the T302A mutant, the proportion of the enzyme in the iron-peroxide state is presumably increased and reactions are favored that utilize this species. In this connection, an investigation by Swinney and Mak (12) on solvent isotope effects on the 17$\alpha$-hydroxylase and 17,20-lyase activities of progesterone 17-hydroxylase-17,20-lyase is particularly relevant. $D_2O$ was found to promote the lytic over the hydroxylation activity, consistent with a greater proportion of the iron-peroxy form of the oxidant being present due to weaker hydrogen ion activity in $D_2O$.

Our results indicate that threonine-302 plays a central role in P450 2B4 in determining the relative rates of various oxygenating reactions catalyzed by this cytochrome. It remains to be established which of the many reactions now known to be catalyzed by P450 require the peroxo-iron intermediate as the direct oxygen donor and which of the many isoforms of this cytochrome make use of the peroxo-iron and oxenoid-iron species as alternative oxygenating agents.

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