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Epoxidation of Olefins by Hydroperoxo−Ferric Cytochrome P450

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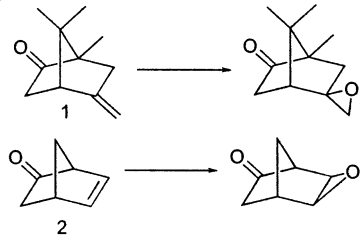
The cytochromes P450 are a superfamily of cysteine thiolate-ligated heme iron enzymes that activate dioxygen for the insertion or addition of a single oxygen atom into a wide variety of substrates, including alkanes to form alcohols, alkenes to form epoxides, sulfides to form sulfoxides, etc.[1,2] P450 enzymes are critical to many biological processes including steroid hormone biosynthesis, drug metabolism, and the detoxification of xenobiotics.[3] The most extensively investigated P450 and the first to be crystallographically characterized[4] is the water-soluble camphor-hydroxylating P450cam from *Pseudomonas putida*.[5] Knowledge of the three-dimensional structure and sequence homology to numerous other cytochromes P450 enabled Sligar, Ishimura, and their co-workers to independently prepare site-specific P450cam mutants in which key amino acids at the active site had been changed.[6,7] We report herein that one such mutant, T252A P450cam, a form of the enzyme that makes little if any hydroxylated product with the normal substrate, 1*R*-camphor, is nonetheless able to catalyze the epoxidation of olefins (Scheme 1). These results directly demonstrate the reactivity of the hydroperoxo−ferric state of P450 in oxygenation reactions.

The reaction cycle of P450 displayed in Scheme 2 has been the object of considerable research for over three decades. The ferric resting, ferric substrate-bound, deoxyferrous, and oxyferrous states (**1**−**4**) have all been thoroughly characterized.[1,2] It has long been thought that addition of the second electron to oxyferrous P450 leads initially to a peroxo−ferric derivative (**5**) that, upon protonation of the distal oxygen, forms a hydroperoxo−ferric intermediate (**6**). A second protonation then leads to O−O bond cleavage with loss of water and generates the "active oxygen" π-cation radical oxoferryl state (**7**). Recently, Hoffman, Sligar, and co-workers were able, using cryogenic irradiation, to reduce oxyferrous D251N, T252A, and wild-type P450 and then use electron paramagnetic and electron nuclear double resonance spectroscopy to directly observe the peroxo and hydroperoxo states and to implicate the involvement of the oxoferryl intermediate in catalysis while also forming the hydroxylated product.[8−10]

While it is generally agreed that oxoferryl P450 (**7**) is the key intermediate in alkane hydroxylations, the possibility of multiple active oxygen species has been discussed in several different systems. Coon and co-workers have proposed hydroperoxo−ferric P450 (**6**) as a second electrophilic oxygen donor in olefin epoxidations.[11,12] Shaik and co-workers have used theory to suggest a two-state reactivity involving both low-spin and high-spin states of oxoferryl P450.[13,14] Jones and co-workers have presented experimental evidence for two different active oxygen species in P450-catalyzed sulfoxidation and N-dealkylation reactions.[15] The fact that T252A P450cam produces hardly any hydroxylated product with camphor as substrate means that it makes very little of the oxoferryl intermediate. Nonetheless, since it accepts electrons from NADH and reduces dioxygen to hydrogen peroxide, it must still make hydroperoxo−ferric P450 (**6** → **2**, path B). Therefore, this P450cam mutant provides an ideal test for the involvement of hydroperoxo−ferric P450 in olefin epoxidation reactions (**6** → **2**, path A). The epoxidation of 5,6-dehydrocamphor and 5-methylenylcamphor by wild-type P450cam has been reported, but the identity of the P450 intermediate responsible for epoxidation was not addressed.[16,17]

Scheme 1. Cytochrome P450 Reactions for Substrates 1 and 2



Scheme 2. Cytochrome P450 Reaction Cycle. States **1**, **2**, and **7** are Neutral, While the Overall Charge on States **3**, **4**, and **6** is −1 and on State 5 is −2

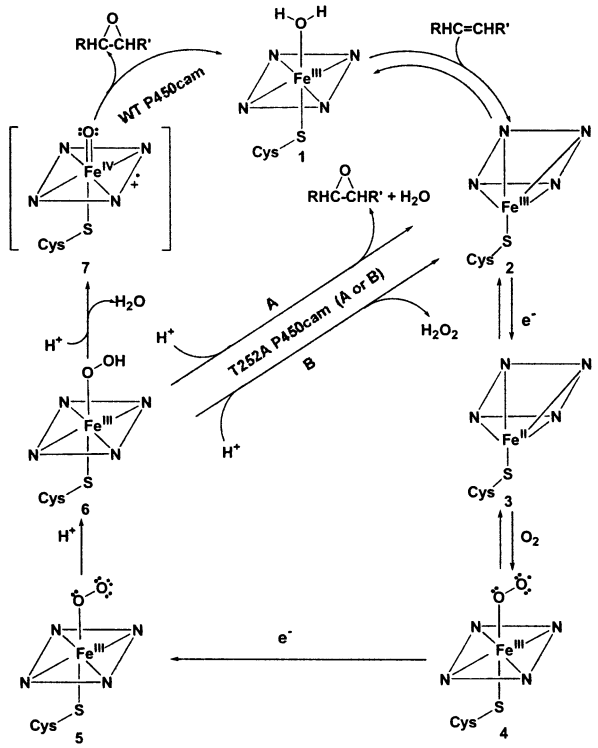


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Table 1. Amounts of Epoxides or Hydroxycam Formed with T252A and Wild-Type P450cam[a]

P450cam	1*R*-camphor	substrate 1	substrate 2
wild type	1200 (ref 23)	98.9 ± 8.7 (4)[c,d]	164.7 ± 17.0 (3)[c]
T252A	0.95 ± 0.1[b] (4)[c]	20.5 ± 3.8 (4)[c]	23.2 ± 7.6 (3)[c]

[a] Product amount (nmol) formed per minute under the conditions described in the text. [b] Data for T252A reactions with camphor are based on a five-minute incubation (divided by five) in order to form enough hydroxycamphor to be quantified by GC. [c] The number of repetitions is indicated in parentheses. [d] This value is in agreement with data previously reported.[23]

Substrates and chromatographic standards were synthesized according to literature procedures.[17−19] For enzymatic reactions, 5-mL reaction vials contained a total volume of 1.0 mL including P450cam (0.5 μM), putidaredoxin (10 μM), substrate (1.0 mM), NADH (1.0 mM), and phosphate buffer (50 mM, pH 7.4, 100 mM KCl). The reactions were initiated by adding putidaredoxin reductase (4.0 μM, 25 μL) and were run at room temperature for 1 min. The reactions were terminated with methylene chloride (0.5 mL), and the products were extracted and analyzed by gas chromatography/mass spectrometry (GC/MS).[20,21] Exo-epoxides were formed almost exclusively.[17,21]

The reactivity of wild-type and T252A P450cam in the hydroxylation of camphor and in the epoxidation of 5-methylenylcamphor and 5-norbornen-2-one is summarized in Table 1. Because T252A P450cam does not hydroxylate camphor, its ability to epoxidize 5-methylenylcamphor was unexpected. For this reason, the reactivity was confirmed both as a function of time and of T252A concentration.[22] The product amounts reported in Table 1 were determined during the time period in which epoxide product was formed linearly. As previously reported, T252A P450cam mainly produces hydrogen peroxide during reaction with camphor. Consequently, hydrogen peroxide, at the same concentration as used for NADH in turnover experiments, was also tested to see if it would epoxidize 5-methylenylcamphor; no product was observed.

T252A P450cam has been extensively investigated in an effort to understand the role played by P450 distal pocket amino acids in the activation of dioxygen. In the cryoreduction experiments of Hoffman and Sligar,[8−10] this mutant was shown to form hydroperoxo−ferric P450 at 77K, just like the wild-type enzyme, but to be unable to make hydroxylated product. Instead, annealing of the species to higher temperatures yielded the ferric resting state without any evidence of product formation. It was concluded that the T252A mutation impairs the delivery of the *second* proton to the distal oxygen of the bound reduced dioxygen species, thereby preventing formation of the active oxygen oxoferryl intermediate. The ability to make the hydroperoxo intermediate without generating the oxoferryl state[8−10] makes this an optimal system with which to test the involvement of the hydroperoxo species in olefin epoxidation. Coon, Vaz, and co-workers have provided initial evidence for hydroperoxo-P450 as an electrophilic oxidant in olefin epoxidation by mammalian P450.[11,12] They reported that Thr to Ala active site mutants of two different P450 isozymes were able to epoxidize styrene and *cis*- and *trans*-2-butene, although in most cases the mutants also produced hydroxylated products, albeit at diminished rates.

As shown in Table 1, T252A P450cam is able to epoxidize the two different olefin substrates examined in the present study. Since this mutant is essentially unable to hydroxylate camphor, it is reasonable to conclude that it does not form the oxoferryl "active oxygen" intermediate. Therefore, epoxidation of olefins by this mutant must be a result of the action of a second oxidant, and the logical identity of that second oxidant is hydroperoxo−ferric P450cam (**6** → **2**, path B), possibly activated by a hydrogen bond or even iron-bound hydrogen peroxide. The present results provide clear-cut confirmation of the proposal by Coon, Vaz, and co-workers that a species such as hydroperoxo−ferric P450 can serve as a second electrophilic oxidant. However, the rate at which this mutant epoxidizes olefins is only about 15−20% of the rate at which wild-type P450cam epoxidizes the same olefins. In addition, less hydroperoxo−ferric P450 will accumulate in wild-type P450cam since it readily converts to the ferryl species. Therefore, although the present results provide convincing evidence that hydroperoxo−ferric P450 is able to epoxidize olefins, it would appear that oxoferryl P450, the consensus active oxygen intermediate for alkane hydroxylation, is also the best catalyst for olefin epoxidation.

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(20) When reactions were run in the presence of catalase and superoxide dismutase, no difference was seen in the amount of product formed.
(21) GC/MS for 5-methylenylcamphor was done using a Finnigan 4500 GC/MS system with a Restek RTX-5 capillary column (30 m × 0.25 mm) with camphor as internal standard. For 5-norbornen-2-one, product was quantified on a DB-17 column with cyclohexene oxide as internal standard.
(22) Reactivity with 5-methylenylcamphor was confirmed both as a function of time and of T252A concentration. Product formation went up linearly with time for a minute and then the rate of formation began to fall off. In the experiment monitoring product formation as a function of T252A concentration, the amount of product formed was proportional to T252A concentration over the range from 0.25 μM to 0.75 μM.
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