Epoxidation of *cis*-1,2-Dideuterio-1-octene by *Pseudomonas oleovorans* Monooxygenase Proceeds without Deuterium Exchange

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Abstract: The mechanism of *Pseudomonas oleovorans* monooxygenase octene epoxidation was examined for possible iron carbene species along the epoxidation pathway. A deuterated olefin substrate (*cis*-1,2-dideuterio-1-octene) was synthesized and the enzymatic product examined for deuterium content. No loss of the deuterium from carbon-2 of the olefin was observed during epoxidation of *cis*-1,2-dideuterio-1-octene by *P. oleovorans* monooxygenase. This indicates that the epoxidation pathway of *P. oleovorans* monooxygenase does not involve the labilization of the hydrogen on carbon-2 of the olefin. We have previously reported that no exchange of deuterium from carbon-1 occurs during *P. oleovorans* monooxygenase epoxidation of either *cis*- or *trans*-1-deuterio-1-octene. Thus, we see no evidence for the involvement of an iron carbene species in the mechanism of octene epoxidation by *P. oleovorans* monooxygenase.

The non-heme iron monooxygenase system from *Pseudomonas oleovorans*, which catalyzes the terminal methyl group hydroxylation of alkanes and fatty acids, was first shown by Coon and co-workers to consist of three protein components: rubredoxin, a flavoprotein reductase, and a non-heme iron monooxygenase.[1] We have shown that this enzyme system is capable of epoxidation of terminal olefins, sulfoxidation of methyl sulfides, and O-demethylation of methyl ethers.[2] Previous work in our laboratory has provided evidence for a chemical mechanism for this enzyme.[2c] Studies with *cis*- and *trans*-1-deuterio-1-octene have shown 70% inversion of olefin configuration and no loss of deuterium from the terminal carbon during epoxidation. To explain these results and the observation that 90% of the epoxide produced from 1,7-octadiene is the *R* enantiomer, we suggested a mechanism in which the oxygen of a presumptive "iron-oxo" species of the enzyme initially attacks carbon-1 of the olefin substrate. Initial oxygen attack at carbon-1 is supported by the exclusive formation of terminal alcohols and terminal epoxides by this monooxygenase system.[1,2] Furthermore, the O-demethylation activity of the monooxygenase system and the observation of aldehyde products during epoxidation reactions also support the view that the initial attack of oxygen occurs at carbon-1 of the olefin.[2c] We proposed that iron-oxo attack at carbon-1 gives rise to a species with cation and/or radical character that closes preferentially from the *si* face of carbon-2, giving rise to the *R* epoxide. The inversion of olefin configuration arises from initial attack being predominantly on the *re* face of the olefin followed by closure from the opposite face to give the epoxide product.[2c]

Cytochrome P-450 is a heme iron monooxygenase system that catalyzes a wide variety of oxidations with a broad substrate specificity.[3] Recently, evidence has been published in support of an epoxidation pathway of cytochrome P-450 that involves formation of an oxametallacyclic species along the reaction pathway.[4] Epoxidation of propene was shown to involve a stereospecific exchange of deuterium from carbon-1 of the olefin. This was interpreted as evidence that the oxametallacyclic species rearranges to an iron carbene at carbon-1, with accompanying exchange of deuterium from the trans position of the olefin. The exchange was only observed for the P-450 system in the presence of P-450 reductase and oxygen, but not with the artificial oxygen donor iodosobenzene.

We have carried out experiments in search of evidence for an iron carbene species along the *P. oleovorans* monooxygenase octene epoxidation pathway, since a mechanism involving such an intermediate could account for the unique inversion of configuration at carbon-1 and retention at carbon-2 that we had observed with deuteriooctene. We anticipated that if an iron carbene intermediate formed at carbon-2 of the octene substrate, this would result in exchange of deuterium at that position. We report herein that no exchange of deuterium was observed from carbon-2 of *cis*-1,2-dideuterio-1-octene during epoxidation by *P. oleovorans* monooxygenase. We have previously reported that no exchange of deuterium from carbon-1 occurs during *P. oleovorans* monooxygenase epoxidation of either *cis*- or *trans*-1-deuterio-1-octene. Thus, we see no evidence for the involvement of an iron carbene species in the mechanism of octene epoxidation by *P. oleovorans* monooxygenase.

Materials and Methods

Enzyme Preparation and Assays. *P. oleovorans* (TF4-1L) was maintained and grown in liquid culture as previously reported.[2b] A partially purified *P. oleovorans* monooxygenase preparation was used for the product accumulations and was prepared as previously reported.[1a] Rubredoxin and *P. oleovorans* reductase were isolated from *P. oleovorans* and purified as previously reported.[1a] In all *P. oleovorans* monooxygenase assays including the large-scale assays the coenzymes and cofactors were prepared as stock solutions with 50 mM tris-HCl buffer (pH 7.4). The following solutions were used: spinach ferredoxin reductase, 1 unit/mL; glucose 6-phosphate (G-6-P) dehydrogenase, 100 units/mL; NADPH, 20 mg/mL; NADH, 20 mg/mL; NAD^+, 20 mg/mL; glucose 6-phosphate, 100 mg/mL.

Large-Scale Enzymatic Product Accumulation and Purification. A typical large-scale product accumulation experiment was performed in a 50-mL flask with a total assay mixture volume of 25 mL. The assay mix contained partially purified *P. oleovorans* monooxygenase (2.0 mL),

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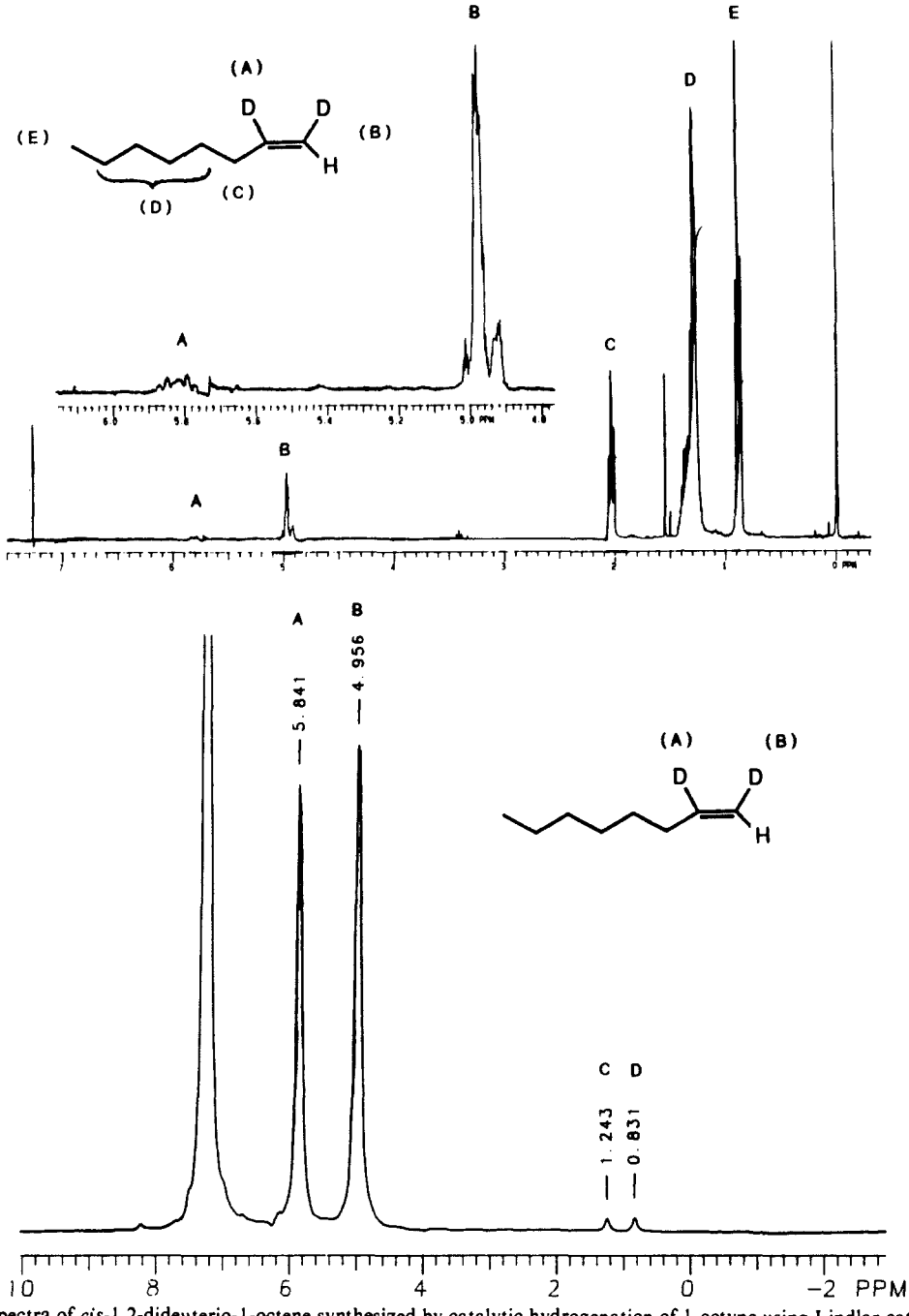


Figure 1. FT NMR spectra of *cis*-1,2-dideuterio-1-octene synthesized by catalytic hydrogenation of 1-octyne using Lindlar catalyst. (A, top) Proton FT NMR, 300 MHz, showing *cis*-1,2-dideuterio-1-octene in $CDCl_3$ with $CHCl_3$ and TMS as chemical shift standards. (B, bottom) Deuterium FT NMR, 400 MHz, showing *cis*-1,2-dideuterio-1-octene in $CHCl_3$ with $CDCl_3$ as a chemical shift standard. Spectrum B also shows some deuterated octane resulting from the catalytic hydrogenation reaction.

rubredoxin (200 μL), spinach ferredoxin reductase (200 μL, 0.200 unit), NADPH (200 μL), G-6-P (200 μL), G-6-P dehydrogenase (200 μL), and the remainder of the volume added as 50 mM tris-HCl buffer (pH 7.4). In some assays, *P. oleovorans* reductase and NADH were substituted for the spinach ferredoxin reductase and NADPH. Substrate (200 μL) was added with acetone (400 μL) as a cosolvent. The assay was shaken in an incubator at 30 °C for 4–6 h. The epoxide product was isolated by extraction into pentane. The pentane extract was washed with saturated $NaHCO_3$ and saturated NaCl and dried over Na_2SO_4. Solvent was removed by evaporation and the product dissolved in a solvent suitable for NMR or GC/MS. A Hewlett-Packard HP 5890 capillary gas chromatograph with a Hewlett-Packard HP 3392A integrator was used for all GC data.

Similar experiments were performed in which the buffer for the assay was prepared with D_2O (99.8 atom % D) and 1-octene was added as the substrate. Dilutions due to the addition of enzyme and cofactor solutions reduced the deuterium content of the final assay solution to approximately 88%. The enzymatic product isolated was characterized by proton NMR and deuterium NMR.

Synthesis of Compounds. *cis*-1,2-Dideuterio-1-octene was synthesized from 1-octyne by catalytic hydrogenation using Lindlar catalyst according to the general method of Lindlar and Dubuis.[5] Palladium on calcium carbonate poisoned with lead (Lindlar catalyst) (0.1 g) and quinoline (1.0 mL, 8.5 mmol) were added to pentane (80 mL) and stirred under an atmosphere of deuterium gas. The 1-octyne (1.5 g, 13.6 mmol) was added through a septum cap and the extent of reaction monitored by GC analysis of aliquots withdrawn by syringe. After 5.5 h the reaction mixture was filtered and the pentane layer washed with water, saturated $NaHCO_3$, and saturated NaCl and dried over Na_2SO_4. Distillation using a short-path apparatus gave *cis*-1,2-dideuterio-1-octene (1.5 mL, 75%). Proton and deuterium NMR data are shown in Figure 1.

The epoxide of *cis*-1,2-dideuterio-1-octene was synthesized from the corresponding olefin by 3-chloroperoxybenzoic acid epoxidation. *cis*-1,2-Dideuterio-1-octene (0.10 g, 0.88 mmol) was added to methylene

(5) Lindlar, H.; Dubuis, R. In *Organic Syntheses*; Baumgarten, H. E., Ed.; Wiley: New York, 1973; Collect. Vol. V, pp 880–883.

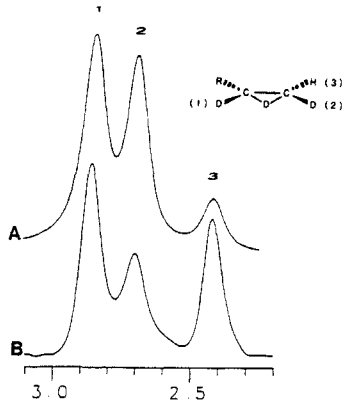


Figure 2. The 400-MHz deuterium FT NMR spectra of the epoxide region of (A) the chemically synthesized (3-chloroperoxybenzoic acid) and (B) the enzymatically generated epoxides of *cis*-1,2-dideuterio-1-octene.

chloride (3.0 mL) containing 3-chloroperoxybenzoic acid (0.16 g, 0.90 mmol) and the reaction stirred for 1 h. The resulting CH_2Cl_2 mixture was washed with saturated Na_2SO_3, water, saturated $NaHCO_3$, and brine and dried over Na_2SO_4. The solvent was removed by evaporation and the product epoxide with unreacted olefin impurity was used without further purification as a standard for GC/MS and NMR studies.

Deuterium NMR Experiments. Both 300- and 400-MHz Varian Gemini FT NMR instruments were used for the FT NMR data. Large-scale enzymatic epoxidations were performed as described above. For NMR measurements, the epoxide was dissolved in $CHCl_3$ and a drop of $CDCl_3$ was included for signal lock on the FT NMR and was used as a reference peak in the spectrum.

Results

cis-1,2-Dideuterio-1-octene was synthesized by catalytic hydrogenation of 1-octyne using deuterium gas. The deuterium content of the product was determined by comparison of the GC/MS data for the *cis*-1,2-dideuterio-1-octene and the protonated standard, 1-octene. By subtraction of the M + 1 and M + 2 contributions of the two next lowest mass peaks from a normalized peak area, the actual percent of nondeuterated, singly deuterated, and doubly deuterated species was calculated. Results of the calculations indicated C_8H_{16} at 0.5%, $C_8H_{15}D$ at 15.3%, and $C_8H_{14}D_2$ at 84.2%. Figure 1 shows the proton and the deuterium FT NMR spectra of the synthesized olefin substrate. The proton FT NMR indicates 0.9 of a deuterium at carbon-2 and 0.8 of a deuterium at carbon-1 of the olefin based on the integrated areas of the peaks in Figure 1. The deuterium FT NMR indicates the same ratio of 9/8 for the deuterium content of carbon-2/carbon-1.

Synthetic 1,2-dideuterio-1,2-epoxyoctane was synthesized from *cis*-1,2-dideuterio-1-octene by using 3-chloroperoxybenzoic acid. The GC/MS data for the synthetic epoxide indicate approximately 85% doubly deuterated and approximately 15% singly deuterated epoxide. This result corresponds to the deuterium content of the starting olefin, *cis*-1,2-dideuterio-1-octene, confirming that no exchange of deuterium occurs during epoxidation with 3-chloroperoxybenzoic acid. Figure 2, trace A shows the deuterium FT NMR spectrum of the epoxide region of the synthetic epoxide from *cis*-1,2-dideuterio-1-octene. As we previously established on the basis of proton relaxation analysis of 7,8-epoxy-1-octene,[2a,d] the NMR peak at 2.4 ppm is the position cis to the alkyl chain in the epoxide of 1-octene. The peak at 2.7 ppm is the position trans to the alkyl chain and the peak at 2.9 ppm is the deuterium on carbon-2 of the epoxide. Comparison of the peak areas indicates that the chemically synthesized epoxide has a deuterium content in the ratio 9/8 for carbon-2/carbon-1. As expected, this corresponds to the relative deuterium contents at carbon-1 and carbon-2 determined from NMR analysis of the starting olefin, *cis*-1,2-dideuterio-1-octene. Comparison of the peak areas for the cis and trans positions at carbon-1 indicates 1/8 deuterium in the position cis to the alkyl and 7/8 deuterium in the position trans to the alkyl in the synthetic 1,2-dideuterio-1,2-epoxyoctane.[6]

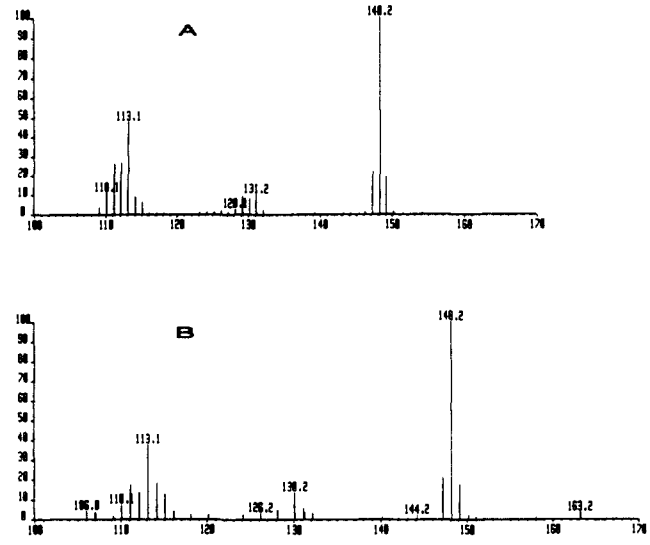


Figure 3. Chemical ionization mass spectra of (A) the chemically synthesized (3-chloroperoxybenzoic acid) epoxide and (B) the enzymatically generated epoxide of *cis*-1,2-dideuterio-1-octene. Chemical ionization using ammonia yields the M + NH_4^+ species at m/e = 148.

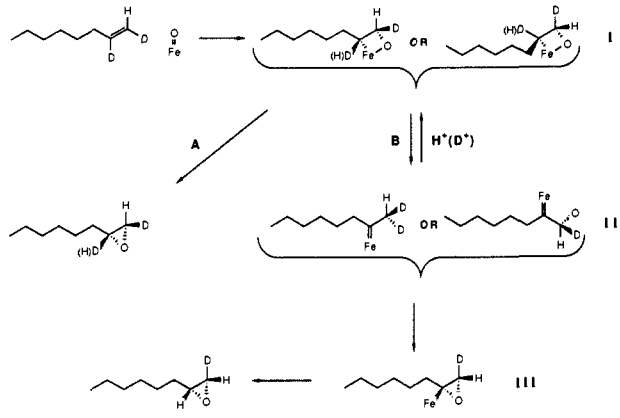
Enzymatic epoxides were obtained from large-scale monooxygenase reactions containing either spinach ferredoxin reductase and NADPH or *P. oleovorans* reductase and NADH, as described in the experimental section. Figure 3 shows a comparison of the chemical ionization GC/MS data for epoxide obtained from the *P. oleovorans* reductase supported reaction and the chemically synthesized epoxide. Ammonia CI was used and the quantitation of deuterium obtained from the M + NH_4^+ (m/e = 148) region of the spectrum. It is evident from Figure 3 that the synthetic and enzymatic epoxides exhibit similar mass spectra, each of which indicates 85% $C_8H_{14}D_2O$, 15% $C_8H_{15}DO$, and a negligible amount of $C_8H_{16}O$. These results correspond with the deuterium content of the starting olefin and establish that the *P. oleovorans* monooxygenase system performs epoxidations without exchange of deuterium on the olefin. Additional enzymatic epoxidation reactions were carried out at higher pH (9.0) as well as at temperatures of 25 and 40 °C; in all cases, mass spectral analysis revealed no loss of deuterium during epoxidation.

Figure 2, trace B shows the deuterium FT NMR of the epoxide region of the enzymatic epoxide of *cis*-1,2-dideuterio-1-octene. Comparison of peak areas indicates that the ratio of deuterium content for carbon-2/carbon-1 in the enzymatic epoxide was 9/8, which corresponds both to the synthetic epoxide data and to the data for the starting olefin, *cis*-1,2-dideuterio-1-octene. The integrated areas of the peaks in Figure 2 were used to calculate the extent of inversion of configuration that occurs during enzymatic epoxidation. Since it is well established that peracid epoxidation proceeds with complete retention of olefin configuration,[2b,7] the relative deuterium content cis and trans to the alkyl at carbon-1 of the synthetic epoxide (Figure 2, trace A) is the same as in the starting olefin. The deuterium content trans to the alkyl in the enzymatic epoxide is equal to the sum of the deuterium content cis to the alkyl in the olefin times the percentage of inversion of olefin configuration during enzymatic epoxidation plus the deuterium content trans to the alkyl times the percentage of retention of olefin configuration during enzymatic epoxidation; a similar relationship holds for the deuterium content cis to the alkyl in the enzymatic epoxide. Calculations carried out in this manner on several samples for either the cis or trans deuterium content

(6) Catalytic hydrogenation reactions normally add hydrogen in a cis orientation to the olefin. However, the last steps in catalytic hydrogenation reactions are known to be reversible, which leads to a small amount of nonspecific exchange of protons. See: March, J. *Advanced Organic Chemistry*, third ed.; John Wiley and Sons: New York, 1985; pp 691–702. This accounts for the small amount of deuterium cis to the alkyl at carbon-1 of the starting olefin.

(7) Berti, G. *Top. Stereochem.* **1973**, *7*, 93–251.

Scheme I. Postulated Mechanism of *Pseudomonas oleovorans* Monooxygenase Which Would Be Analogous to the Mechanism Proposed for P-450[a]



[a] For clarity only two of the possible products from this mechanism are illustrated in the scheme.

indicated approximately 70% inversion of olefin configuration occurs during epoxidation by the *P. oleovorans* monooxygenase system. This result is in agreement with our previous studies with olefins deuterated at carbon-1 in the cis or trans positions, from which we concluded that enzymatic epoxidation proceeds with 70% inversion of olefin configuration and with no loss of deuterium from carbon-1.

It is clear from the above results with deuterated substrate that no loss of deuterium is detectable during the *P. oleovorans* monooxygenase catalyzed epoxidation reaction. In order to test for possible incorporation of solvent deuterium into the epoxide products, *P. oleovorans* monooxygenase catalyzed epoxidations of 1-octene were carried out in D_2O. Enzyme reactions using 1-octene as the substrate and D_2O as the reaction solvent resulted in an epoxide product that showed the expected peaks in the proton FT NMR spectra and no deuterated epoxide peaks in the deuterium FT NMR spectra. Also, GC/MS data indicated no deuterium incorporation in the epoxide product. These results confirm that epoxidation proceeds with no exchange of deuterium at carbon-2.

Discussion

In previously published results from this laboratory, evidence was presented that the initial attack by the oxygen of the putative iron–oxo species of *P. oleovorans* monooxygenase occurs at carbon-1 of the olefin.[2c] This was supported by the observation of absolute specificity for terminal methyl hydroxylation of alkanes and by the absolute specificity for epoxidation of only terminal olefins by the *P. oleovorans* monooxygenase system. Furthermore, the *P. oleovorans* monooxygenase system performs O-demethylation of methyl ethers and produces aldehyde as a second product during the epoxidation reaction. Ketone was not observed, as would have been expected for oxygen attack at carbon-2 of the olefin. If the oxametallacyclic mechanism proposed for P-450[4] is adapted to provide for the accumulated evidence describing the *P. oleovorans* monooxygenase system, the mechanism in Scheme I would be anticipated. This mechanism is identical with that proposed for P-450 except that the olefin approaches the iron–oxo species in the orientation required for oxygen attack at the terminal carbon. By analogy to the mechansim proposed for P-450 there are two possible pathways to the epoxide. The enzymatic epoxidation reaction would be postulated to partition between the pathways in this mechanism in order to account for the stereochemistry at carbon-2 and 70% inversion of olefin configuration that are observed in the product epoxide.[2c]

With this postulated mechanism in mind, experiments were undertaken to look for deuterium exchange at the second carbon of 1-octene during epoxidation by the *P. oleovorans* monooxygenase system. *cis*-1,2-Dideuterio-1-octene was synthesized and the enzymatic product was analyzed by GC/MS and deuterium FT NMR. No exchange of deuterium was observed at carbon-2 based on the GC/MS data. This result represents strong evidence against the intermediacy of the iron carbene species, II, in octene epoxidations catalyzed by the *P. oleovorans* monooxygenase system.

It is important to point out that this conclusion is independent of our presumption that the oxygen of the putative iron–oxo species of the enzyme attacks the terminal carbon of the olefin substrate. If we consider the alternate possibility that the olefin approaches the iron–oxo species so that the oxygen attacks at carbon-2 of the olefin, the mechanism of Scheme I would predict exchange of deuterium from carbon-1 of the olefin during enzymatic epoxidation. We have previously reported that no exchange of deuterium from carbon-1 occurs during *P. oleovorans* monooxygenase epoxidation of either *cis*- or *trans*-1-deuterio-1-octene.[2c] Therefore, our results are inconsistent with the presence of an iron carbene species at either carbon-1 or carbon-2 of the olefin substrate in the mechanism of octene epoxidation by the *P. oleovorans* monooxygenase system.

It might be postulated that if a base at the *P. oleovorans* monooxygenase active site were capable of sequestering the deuterium from species I, this deuterium might be redonated to substrate during protolysis of the iron–carbon bond in the last step of pathway B. However, the active site of *P. oleovorans* monooxygenase is capable of binding and oxidizing a wide variety of substrates including 2-methoxy-1-octene,[2e] 4-(2-methoxyethyl)-phenyl allyl ether,[8] and allylanisole.[9] These results with bulky substituents near the position of oxidation on the substrate suggest that the active site is sufficiently open to allow exchange of protons by species formed during catalysis. Furthermore, we have carried out enzymatic epoxidation at several temperatures (as well as at pH 9) and no deuterium exchange was evident from mass spectral analyses of the product epoxides. This contrasts with the well-studied case of glyoxalase, where a fast proton-transfer mechanism via an enediol at a highly protected active site gives rise to an increase in solvent proton incorporation as the temperature is increased from 25 to 35 °C.[10] Finally, such a sequestering is *not* observed in propene epoxidation by P-450, where 95% exchange of deuterium is observed and is postulated to arise from the protolysis of the iron carbene species. Thus, while we cannot definitively rule out the possibility of a sequestered proton, we consider this possibility to be highly unlikely.[11]

Taken together, the results presented herein and previously reported mechanistic studies with the *P. oleovorans* monooxygenase system are consistent with the mechanistic view of octene epoxidation presented previously.[2c] While our results are inconsistent with an iron carbene, we note that an oxametallacyclic species can be viewed as a resonance structure for species with cation and/or radical character, which we have previously suggested to be involved in the *P. oleovorans* monooxygenase octene epoxidation mechanism.

Acknowledgment. We gratefully acknowledge the support of the National Institutes of Health (GM 39350).

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(10) Hall, S. S.; Doweyko, A. M.; Jordan, F. *J. Am. Chem. Soc.* **1976**, *98*, 7460–7461.

(11) Groves et al.[4b] rationalized the lack of exchange seen with longer chain olefins on the basis of the topology of the P-450 active site restricting "orientation of the intermediates, thus influencing the ability of an amino acid side chain base to deprotonate the metallacycle". While this argument may be valid for the case of P-450, we note that since for the *P. oleovorans* monooxygenase an iron carbene would probably be expected to form at carbon-2, the steric sensitivity of the mechanism might be different from the case of P-450. In any event, no structural information is available regarding the topology of the *P. oleovorans* monooxygenase active site; in the absence of such information, we cannot extrapolate from our octene results to substrates of different structure.