# Mechanistic Studies on Non-Heme Iron Monooxygenase Catalysis: Epoxidation, Aldehyde Formation, and Demethylation by the ω-Hydroxylation System of *Pseudomonas oleovorans*

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**Abstract:** In previous work we have established that the "ω-hydroxylation" system of *P. oleovorans* readily converts terminal olefins to the corresponding 1,2-oxides and does so stereoselectively. We also demonstrated loss of olefin configuration during enzymatic epoxidation, a result inconsistent with a concerted epoxidation mechanism (*J. Am. Chem. Soc.* **1977**, *99*, 2017–2024). Since loss of olefin configuration is unprecedented for monooxygenase-catalyzed epoxidations, these studies have been confirmed with isolated enzymes and further extended in order to probe the mechanism of non-heme iron monooxygenase catalysis. Enzymatic epoxidation of both *cis*- and *trans*-1-deuterio-1-octene proceeds with about 70% inversion of the olefinic configuration, with corresponding results being obtained for the two olefins. As we reported in a preliminary communication (*Bio/Technology* **1983**, *1*, 677–686), the ω-hydroxylation system also produces aldehydes from olefins. Aldehyde formation exhibits the reaction characteristics expected for the usual oxygenase pathway. Deuterium migration from C-1 to C-2 occurs in formation of aldehyde from olefin, although loss of deuterium also occurs. The ω-hydroxylation system was found to efficiently catalyze O-demethylation of heptyl methyl ether, the first demonstration of such activity for a non-heme iron monooxygenase of this type. Taken together, the results provide support for a two-step mechanism involving enzyme-generated species with cationic and/or radical character, which accounts for the stereoselectivity, configurational loss, substrate specificity, formation of aldehydes with deuterium migration, and demethylation activity exhibited by this enzyme system.

The mechanism by which oxygenases catalyze the insertion of molecular oxygen into organic molecules has been the subject of intense scrutiny in recent years, due to the importance of these enzymes in detoxification, oncogenesis, biosynthesis, and metabolism in general. However, while the P-450 and the flavin-containing monooxygenases have been intensively studied at the molecular level, the state of our understanding of the molecular basis of non-heme iron monooxygenase catalysis is, by comparison, poor indeed. Non-heme iron monooxygenases comprise a large class of enzymes which, in addition to the *P. oleovorans* system discussed here, includes the phenylalanine, tyrosine, and tryptophan monoxygenases as well as squalene epoxidase.[1,2]

The non-heme iron monooxygenase system from *P. oleovorans* which catalyzes 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 non-heme iron monoxygenase.[3-13] In previous work from this laboratory, we demonstrated that this enzyme system readily converts terminal olefins to the corresponding 1,2-epoxides, and does so stereoselectively, producing the R-(+) epoxides.[14-18] We have also examined the specificity of this reaction[19,20] and have carried out immobilization, metal substitution, and chemical modification experiments with rubredoxin.[21-23] However, the instability of the monooxygenase hampered detailed mechanistically oriented experiments with the isolated system, a common problem with the non-heme iron monooxygenases. We therefore turned to the use of resting whole cells and demonstrated through the use of the substrate *trans,trans*-1,8-dideuterio-1,7-octadiene that enzymatic epoxidation does not proceed with retention of the original olefin geometry.[24] Taken together, the combined stereoselectivity of oxygen attack at carbon 2 and lack of configurational retention at carbon 1 are inconsistent with a concerted "oxenoid" mechanism, and we suggested possible two-step mechanisms involving cationic and/or radical intermediates.

The goals of the present study were to confirm and extent these mechanistic studies using purified reductase and rubredoxin and a partially purified preparation of the monooxygenase. We wished, first of all, to demonstrate loss of olefin configuration with both *cis*- and *trans*-deuterated substrates and to establish whether complementary ratios of *cis*- and *trans*-epoxides would be obtained from these two substrates. This was an important goal, since the

(1) Comprehensive reviews on oxygenases can be found in: (a) "The Enzymes", 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1975; vol. 12. (b) "Molecular Mechanisms of Oxygen Activation"; Hayaishi, O., Ed.; Academic Press: New York, 1974. Also, for a review of biological hydroxylation reactions, see: (c) Gunsalus, I. C.; Pederson, T. C.; Sligar, S. G. *Annu. Rev. Biochem.* **1975**, *44*, 377–407. (d) A review on P-450 oxygenation in: White, R. E.; Coon, J. J. *Annu. Rev. Biochem.* **1980**, *49*, 315–356.

(2) For a recent review on enzymatic epoxidation reactions, see: May, S. W. *Enzyme Microb. Technol.* **1979**, *1*, 15–22.

(3) Peterson, J. A.; Basu, D.; Coon, M. J. *J. Biol. Chem.* **1966**, *241*, 5162–5164.

(4) Peterson, J. A.; Kusunose, M.; Kusunose, E.; Coon, M. J. *J. Biol. Chem.* **1967**, *242*, 4334–4340.

(5) Peterson, J. A.; Coon, M. J. *J. Biol. Chem.* **1968**, *243*, 329–334.

(6) McKenna, E. J.; Coon, J. J. *J. Biol. Chem.* **1971**, *245*, 3882–3889.

(7) Lode, E. T.; Coon, J. J. *J. Biol. Chem.* **1971**, *246*, 791–802.

(8) Ueda, T.; Lode, E. T.; Coon, J. J. *J. Biol. Chem.* **1972**, *247*, 2109–2116.

(9) Benson, A.; Tomoda, K.; Chang, J.; Matsueda, G.; Lode, E. T.; Coon, M. J.; Yasunogu, K. T. *Biochem. Biophys. Res. Commun.* **1971**, *42*, 640–646.

(10) Boyer, R. R.; Lode, E. T.; Coon, M. J. *Biochem. Biophys. Res. Commun.* **1971**, *44*, 925–930.

(11) Ueda, T.; Coon, M. J. *J. Biol. Chem.* **1972**, *247*, 5010–5016.

(12) Ruettinger, R. T.; Olson, S. T.; Boyer, R. F.; Coon, M. J. *Biochem. Biophys. Res. Commun.* **1974**, *57*, 1011–1017.

(13) Ruettinger, R. T.; Griffith, G. R.; Coon, M. J. *Arch. Biochem. Biophys.* **1977**, *183*, 528–537.

(14) May, S. W.; Abbott, B. J. *Biochem. Biophys. Res. Commun.* **1972**, *48*, 1230–1234.

(15) May, S. W.; Abbott, B. J. *J. Biol. Chem.* **1973**, *248*, 1725–1730.

(16) May, S. W.; Schwartz, R. D. *J. Am. Chem. Soc.* **1974**, *96*, 4031–4032.

(17) May, S. W.; Steltenkamp, M. S.; Schwartz, R. D.; McCoy, C. J. *J. Am. Chem. Soc.* **1976**, *98*, 7856–7858.

(18) May, S. W. *Catal. Org. Synth.* [*Conf.*] *4th, 1976* **1977**, 101–111.

(19) May, S. W.; Schwartz, R. D.; Abbott, B. J.; Zaborsky, O. R. *Biochem. Biophys. Acta* **1975**, *403*, 245–255.

(20) May, S. W.; Steltenkamp, M. S.; Boram, K. R.; Katopodis, A. G.; Thowsen, J. R. *J. Chem. Soc., Chem. Commun.* **1979**, 845–846.

(21) May, S. W.; Kuo, J. Y. *J. Biol. Chem.* **1977**, *252*, 2390–2395.

(22) May, S. W.; Kuo, J. Y. *Biochemistry* **1978**, *17*, 3333–3338.

(23) May, S. W.; Lee, L. G.; Katopodis, A. G.; Kuo, J. Y.; Wimalasena, K.; Thowsen, J. R. *Biochemistry* **1984**.

(24) May, S. W.; Gordon, S. L.; Steltenkamp, M. S. *J. Am. Chem. Soc.* **1977**, *99*, 2017–2024.

loss of olefin configuration during epoxidation is, to our knowledge, unprecedented for any other monooxygenase system described in the literature. Second, as we have recently reported,[25] preliminary studies with the purified *P. oleovorans* monooxygenase (POM) indicated formation of aldehydes, in addition to epoxides, from terminal olefins. Since aldehyde formation could arise as a consequence of group migration in an enzymatically produced species with cationic character, we wished to examine the aldehyde formed from 1,1-dideuterio-1-octene in order to obtain direct evidence for intramolecular rearrangement. Finally, in order to obtain further evidence regarding the locus of initial oxygen attack, the possibility that POM could catalyze demethylation reactions with appropriate substrates was investigated. The results reported herein have allowed us to propose a mechanism of oxygenation by this non-heme iron monooxygenase, which is consistent with all stereochemical and mechanistic results so far reported.

## Experimental Section

Hydrocarbons, solvents, organic reagents, and standards were purchased from various sources and were of the highest purity available. Authentic epoxide standards were synthesized by using *m*-chloroperbenzoic acid and the appropriate olefins, as described previously.[15] *P. oleovorans* cultures were grown on *n*-octane under conditions described elsewhere.[24] *P. oleovorans* rubredoxin was purified by using a procedure described by us earlier.[21,22] Spinach ferredoxin-NADP^{+} reductase, glucose-6-phosphate dehydrogenase, and all other biochemicals were purchased from Sigma. Reaction conditions for standard product-formation assays as well as procedures for extraction and quantitation of products were as described, except in some cases where, as indicated, the extraction solvent was changed to facilitate extraction and concentration of the various products.

**Synthesis of *trans*-1-Deuterio-1-octene.** The procedure developed by Brown and Gupta was used.[26] The crude product was distilled, bp 121 °C (760 mm), and further purified by preparative GC (20-ft carbowax 20 M, 90 °C). The final product consisted of a single peak with retention identical with the fully protonated 1-octene standard. The NMR was consistent with the expected structure and showed 94% deuterium in the trans position, and the cis isomer was not detectable.

**Synthesis of 1-Deuterio-1-octyne.** A solution of 11 g (100 mmol) of freshly distilled 1-octyne in 100 mL of anhydrous ether was cooled to 0 °C in an ice-salt bath and 1.1 equiv of freshly titrated methyllithium in ether (purchased from Aldrich) was added dropwise under argon. The reaction mixture was stirred 3 h at 0 °C and allowed to gradually warm to room temperature. $D_2O$, 3.0 g (100% D), was then added, stirred for $^1/_2$ h at room temperature, and neutralized with 10% acetic acid. The reaction mixture was poured into ice-cold water and extracted thrice with 25-mL aliquotes of ether, and the combined extracts were washed with saturated $NaHCO_3$ and saturated brine, dried over anhydrous $MgSO_4$, and distilled to give 9 g of 1-deuterio-1-octyne (bp 125 °C (760 mm)). NMR analysis indicated that the product was 1-deuterio-1-octyne with only 80% deuterium incorporated. The material was again reacted with methyllithium and $D_2O$, and after distillation 6 g of material was obtained with 99% D incorporation (NMR). Gas chromatographic analysis indicated that the product consisted of one peak with retention time identical with a 1-octyne standard.

**Synthesis of 1,1-Dideuterio-1-octene.** 1-Deuterio-1-octyne, 5 g, was reacted with catechol borane and then deuterated acetic acid as indicated for 1-deuterio-1-octene. The olefin was isolated by preparative gas chromatography, and NMR analysis showed 99% deuterium in the cis position and 86% deuterium in the trans position.

**Synthesis of *cis*-1-Deuterio-1-octene.** 1-Deuterio-1-octyne, 5 g, was reduced as above, using protioacetic acid in the final step. The resulting product exhibited one peak coeluting with 1-octene on the GC. Mass spectral analysis was consistent with 1-deuterio-1-octene, and NMR analysis indicated that 99% deuterium was present in the cis position and no trans was detectable.

**Synthesis of *trans*-1,2-Epoxy-1-deuteriooctane, *cis*-1,2-Epoxy-1-deuteriooctane, and 1,2-Epoxy-1,1-dideuteriooctane.** A solution of each olefin (0.06 mL, 0.38 mmol) and *m*-chloroperbenzoic acid (100 mg, 0.58 mmol) in 14 mL of $CH_2Cl_2$ was stirred at room temperature overnight, after which it was diluted with 25 mL of $CH_2Cl_2$, washed with sodium bisulfite and $NaHCO_3$, and then dried over anhydrous $MgSO_4$. The concentrated product was purified by preparative gas chromatography to obtain high-purity epoxides.

**POM Partial Purification.** The procedure described here is a modification of processes published elsewhere[13] and of our previous procedures[27] and affords a form of enzyme stable and active enough to produce milligram quantities of products. Two hundred grams of *P. oleovorans* cells were lysed for 2 h in 1.5 L of 20 mM Tris Cl, pH 7.4 (all operations were performed at 4 °C, and the pH was adjusted at every step). The solution was centrifuged and the membrane pellet was lysed again for another 2 h. After centrifugation the membrane pellet was resuspended in 600 mL of the same buffer and sonicated in 1-min blasts for a total of 5 min with a Branson Sonifier. The solution was diluted with 600 mL of cold buffer and centrifuged (20 000 g, 1 h), and the supernatant was subjected to ammonium sulfate precipitation. The 30–35% pellet was resuspended in 50 mL of 20 mM Tris Cl, 20% glycerol, 0.1% deoxycholate, pH 7.4 buffer and centrifuged again (50 000 g, 2 h), and the supernatant was assayed and stored at –70 °C. The best preparations had a specific activity of 465 nmol per mg of protein of total (both epoxide and aldehyde) product in the standard GC assay with 1,7-octadiene as a substrate.[15,24]

**Assays.** In a total volume of 1 mL of buffer (100 mM Tris Cl, pH 7.4) were added 1 mg of partially purified *P. oleovorans* monooxygenase, 80 μg of *P. oleovorans* rubredoxin, 14 μg spinach ferredoxin reductase, 0.5 mg NADPH and 20 μL of substrate solution (100 μL/1.00 mL of acetone). The mixture was shaken at 25 °C for 10 min. It was then extracted with 200 μL of hexane by using 2-octanol as the internal standard, and the hexane layer was analyzed by GC. Whenever NADPH recycling was needed 2 units of glucose-6-phosphate dehydrogenase and 2 mg of glucose 6-phosphate were included in the incubation mixture.

The crude system was obtained by suspending 100 g of cells in 300 mL of buffer. The solution was sonicated for 7 min in 1-min blasts. It was then centrifuged (20 000 g, 1 h), and the cloudy supernatant was again centrifuged (35 000 g, 2 h) to obtain a clear yellow-pink solution. This clarified cell extract was very stable upon freezing and was assayed by adding 0.20 mL of cell extract to 0.80 mL of buffer and 20 μL of substrate solution. The assay mixture was then incubated and extracted as for the partially purified system.

Whenever detection of carboxylic acids was required, after the incubation time was completed the assay mixture was basified to pH >12 and washed with an equal volume of pentane, and the mixture was then acidified to pH <2 and extracted with an equal volume of ether. The ether extract was dried, evaporated to dryness, and then reacted with $BF_3$/MeOH.

**Enzymatic Reactions.** Products from the enzymatic reaction of the different substrates were obtained as follows: In a total volume of 20 mL of buffer (100 mM Tris Cl, 20% glycerol, 0.1% deoxycholate, pH 7.4) were added 3 mg of the POM sample, 1 mg of *P. oleovorans* rubredoxin, 0.2 mg of spinach ferredoxin reductase, 100 units of glucose-6-phosphate dehydrogenase, 200 mg of glucose 6-phosphate, 1 mg of NADPH, and 200 μL of substrate in 2.00 mL of acetone. The mixture was incubated at 25 °C for 30 min with additional substrate and glucose 6-phosphate being added after 15 min incubation time. The mixture was then extracted with cold pentane 3 times, and the combined pentane extracts were dried over $MgSO_4$ and concentrated. The procedure was repeated for every substrate, and the various products were isolated by preparative gas chromatography. Product yield was increased somewhat if the reaction vessel was stoppered with a septum and a needle connected to an oxygen line with a slight positive pressure was inserted. This method minimized loss of substrates and products by evaporation and was used in some cases. In order to obtain milligram quantities of the aliphatic acid products, the same procedure was repeated, using only 10 units of glucose-6-phosphate dehydrogenase and allowing the incubation to proceed to 1 h. The incubation mixture was then extracted in a manner similar as for the equivalent assay mixture.

**NMR Procedures and Instrumentation.** The enzymatic products (0.1–2 mg) were dissolved in 0.25 mL of $CCl_4$ containing 10% (v/v) $C_6D_6$, in a 5-mm high-resolution NMR sample tube. Proton FT NMR spectra were obtained at 300.13 MHz using a Bruker WM-300 FTNMR spectrometer equipped with an ASPECT-2000 computer system. Areas of the relevant spectral features were measured with a Gelman compensating polar planimeter and were reproducible to better than 10%.

**GC–MS Analysis.** Enzymatic products and standards were analyzed with a Varian 3700 gas chromatograph fitted with a DB-1 bonded phase, fused silica open tubular column (30 m × 0.25 mm) purchased from J & W Scientific Co., Rancho Cordoba, CA. The gas chromatograph was interfaced with a Varian MAT 112S mass spectrometer with a Varian MAT SS200 data system. The operating temperatures were injector 230 °C, detector 230 °C, GC/MS interface 240 °C, and column oven 100 °C programmed at 5 deg/min to 250 °C. The injections were made by

(25) May, S. W.; Padgette, S. R. *Bio/Technology* **1983**, *23*, 2187–2192. *1*, 677–686.

(26) Brown, H. C.; Gupta, S. K. *J. Am. Chem. Soc.* **1975**, *97*, 5249–5255.

(27) Katopodis, A. G. Ph.D. Thesis, Georgia Institute of Technology, Atlanta, 1982.

**Table I**[a]

| substrate | products[b] |
|---|---|
| **Crude System**[c] | |
| octane | 1-octanol |
| 1,7-octadiene | 7,8-epoxy-1-octene |
| 1-octene | 1,2-epoxyoctane |
| | 1-octen-8-ol |
| **Purified System**[d] | |
| octane | 1-octanol |
| 1,7-octadiene | 7,8-epoxy-1-octene |
| | 1-octen-8-al |
| 1-octene | 1,2-epoxyoctane |
| | 1-octanal |
| | 1-octen-8-ol |
| **Partially Purified System with NADPH Recycling**[e] | |
| octane | 1-octanol |
| | 1-octanal |
| 1,7-octadiene | 7,8-epoxy-1-octene |
| | 1-octen-8-al |
| 7,8-epoxy-1-octene | no product[f] |
| 1-octene | 1,2-epoxyoctane |
| | 1-octanal |
| | 1-octen-8-ol |
| 1,2-epoxyoctane | no product[f] |

[a] All assays were performed by our standard GC method. The table lists products formed after 10 min of incubation at 25 °C. [b] The products reported here do not include any of the acids to which the alcohols and aldehydes are metabolized. These acids are not detected in our standard assay conditions unless esterified (see text). [c] These assays were performed using clarified, sonicated whole cells. [d] Purified POM was obtained by the method described elsewhere.[26] The assays were performed using spinach ferredoxin reductase and purified *P. oleovorans* rubredoxin. [e] The partially purified POM was assayed by using spinach ferredoxin reductase and purified *P. oleovorans* rubredoxin. Glucose-6-phosphate dehydrogenase and glucose 6-phosphate were added to recycle the NADPH used by the monooxygenase. [f] Although diepoxide or epoxy ol products may be formed as described previously,[14,15,19] these are not detectable in the 10-min incubation time used in these experiments.

using the Grob splitless injection technique, and the mass spectrometer was operated at resolution 600, electron energy 80 eV, and source temperature 220 °C.

**Results**

Table I illustrates the reactivity pattern exhibited by the POM system at three stages of purity. As we have indicated in previous work, the crude system produces terminal alcohols from alkanes and epoxides from terminal olefins. The purified POM system, on the other hand, produces aldehydes from terminal olefins in addition to the expected products. It must be noted that although recently we have been able to purify and partially stabilize this monooxygenase,[27] our goal was to prepare milligram quantities of enzymatic products, and the purified system presented many difficulties during scale-up. We therefore turned to an intermediate system, a partially purified preparation. The 30–40% ammonium sulfate fraction prepared from sonicated cell membranes exhibited excellent stability and a reactivity pattern similar to that of the crude system, when reconstituted with purified reductase and rubredoxin. When, on the other hand, an efficient NADPH recycling system was included in the reaction mixture, the products produced by the partially purified system resembled those obtained from the purified system. Thus, as shown in Table I, with NADPH recycling, aldehydes are detected as products of the partially purified POM. Control experiments showed that without cofactor recycling, the partially purified POM produced octanoic acid from 1-octene and 7-octenoic acid from 1,7-octadiene, as established by acidification of the assay mixture, extraction with ether, and conversion to the methyl esters. In contrast, formation of neither acid was detected in the presence of the NADPH recycling system, where the aldehydes accumulate. It is thus apparent that POM produces both epoxides and aldehydes from terminal olefins, but the aldehydes are efficiently transformed to acids by contaminating enzymes which are $NADP^+$
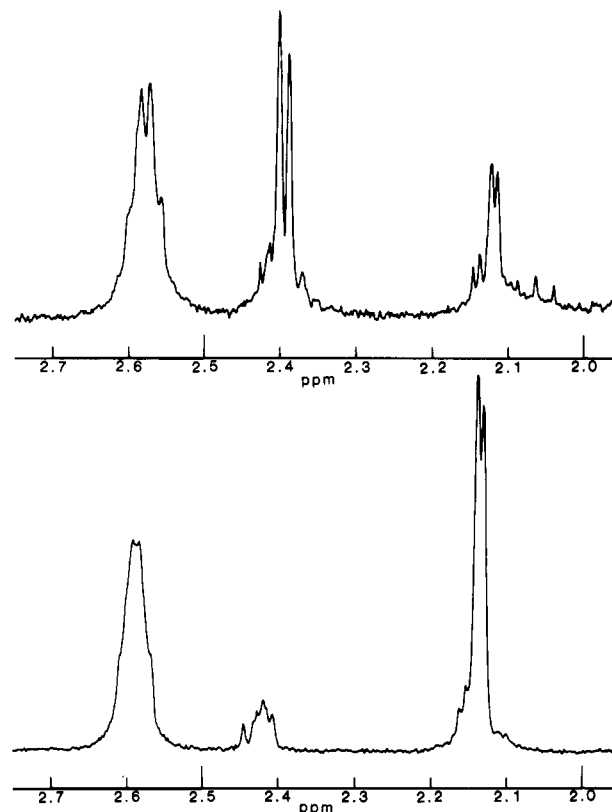


**Figure 1.** FTNMR spectra, 300 MHz, of the epoxide region of enzymatically and chemically generated epoxides from *trans*-1-deuterio-1-octene. The 300-MHz FTNMR spectrum of the epoxide proton region of (a) the epoxide obtained enzymatically from *trans*-1-deuterio-1-octene (top panel) and (b) *trans*-1,2-epoxy-1-deuteriooctane obtained by the chemical (*m*-chloroperbenzoic acid) oxidation of the *trans*-1-deuterio-1-octene sample used for the enzymatic epoxidation reaction (bottom panel). (Note the presence of about 6% of undeuterated epoxide due to the undeuterated olefin in the starting material.)

dependent. These fatty acids are not detectable by the extraction and GC procedure used for routine detection of epoxides and aldehydes.

**Configurational Studies on Epoxides.** Enzymatically produced epoxides from deuterated substrates were isolated by preparative gas chromatography and their proton FTNMR spectra were analyzed as indicated in the Experimental Section. Figures 1, 2, and 3 show the FTNMR spectra of the epoxide proton region of epoxides obtained from *trans*-, *cis*-dideuterio-1-octene, and dideuterio-1-octene, respectively, both enzymatically and via *m*-chloroperbenzoic acid epoxidation. It is evident from the spectra that *trans*-1-deuterio-1-octene gave rise to 1,2-epoxyoctane with 72% cis deuterium and 28% trans deuterium. In the complementary case, *cis*-1-deuterio-1-octene produced 1,2-epoxyoctane with 70% trans deuterium and 30% *cis* deuterium. Therefore our results with the partially purified system are in excellent agreement with our previous results using cell suspensions.[24] They are also consistent with our previous demonstration that no isomerization of either the epoxide or olefin functionalities occurs under the reaction conditions or during GC isolation. Furthermore, GC-MS analysis (chemical ionization) of the enzymatically produced epoxide from 1,1-dideuterio-1-octene showed an $M^+ + 1$ peak at $m/z$ of 131 and no detectable peaks at 129 or 130, indicating that all the deuterium present in the substrate is also present in the epoxide product.

Taken together, these results confirm and extend our previous report that epoxidation by POM does not proceed with retention of configuration, a finding that is to our knowledge unprecedented. The clear implication of these results is that epoxidation proceeds via a stepwise mechanism involving cationic and/or radical species.

**Aldehyde Products.** In our attempt to obtain direct evidence that aldehydes are produced from olefins by POM via an intra-
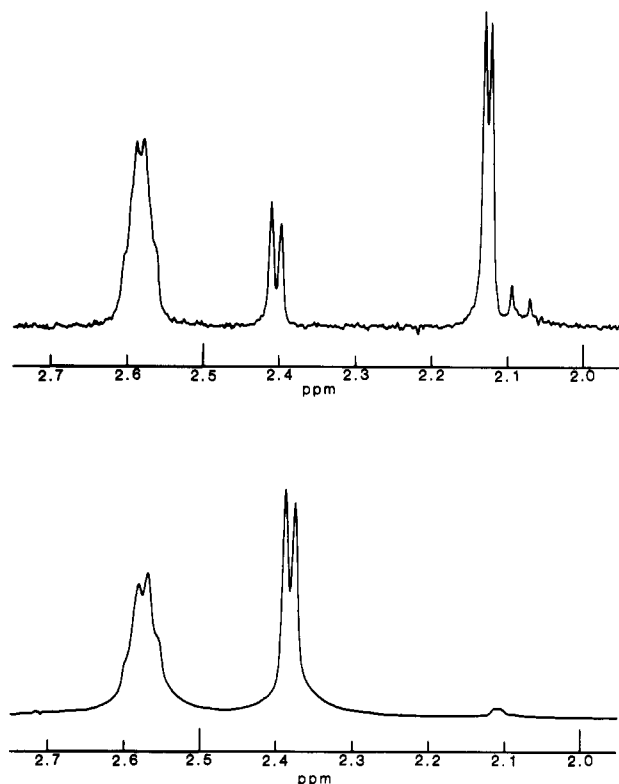
**Figure 2.** FTNMR spectra, 300 MHz, of the epoxide region of enzymatically and chemically generated epoxides from *cis*-1-deuterio-1-octene. The 300-MHz NMR spectrum of the epoxide proton region of (a) the epoxide obtained enzymatically from *cis*-1-deuterio-1-octene (top panel) and (b) *cis*-1,2-epoxy-1-deuteriooctane obtained by the chemical (*m*-chloroperbenzoic acid) oxidation of the *cis*-1-deuterio-1-octene sample used for the enzymatic epoxidation reaction (bottom panel). (Note the presence of a small amount of undeuterated epoxide due to the undeuterated olefin in the starting material.)

molecular group migration process, we wished to examine the aldehyde produced enzymatically from 1,1-dideuterio-1-octene, anticipating the possibility of deuterium migration to carbon 2 of the aldehyde product. However, when the aldehyde product was isolated from a large-scale incubation and examined by GC-MS and FTNMR, we found that extensive loss of deuterium from carbon 2 occurred. Since this could be the result of exchange—either under the reaction conditions or during GC-MS analysis—we performed another set of large-scale incubations with the NADPH recycling system omitted, in order to allow efficient conversion of the aldehyde to the corresponding acid by the aldehyde dehydrogenase present in our preparations. The octanoic acid thus produced from 1,1-dideuterio-1-octene was isolated and reacted with $BF_3/MeOH$ to give the corresponding methyl ester. GC-MS analysis of the methyl octanoate clearly indicated the presence of molecules containing one deuterium on carbon 2. This is evident from the electron impact spectrum which shows a base peak at $m/z$ 75 arising from the diagnostic McLafferty rearrangement of the molecular ion, indicating that deuterium is present on the carbon 2. In addition, the peak at $m/z$ 128 arising from $RCHDCO^+$ (loss of $OCH_3$) confirms this conclusion. By comparison, the mass spectrum of protiomethyl octanoate shows the corresponding peaks at $m/z$ 74 and 127. Finally, selective ion monitoring of the chemical ionization spectrum of the enzymatic product indicated the presence of species with an $M^+ +$ 1 peak at $m/z$ 160, corresponding to the deuterated methyl octanoate. In addition, a substantial amount of a species with $M^+$ + 1 at $m/z$ 159, corresponding to the protio ester was also present. Similar results were obtained from GC–MS analysis of the octanoic acid produced from 1,1-dideuterio-1-octene by POM of hexane grown cells. We note also that a small amount of octanoic acid is present in POM preparation, and preliminary experiments indicate that it is essential for activity.
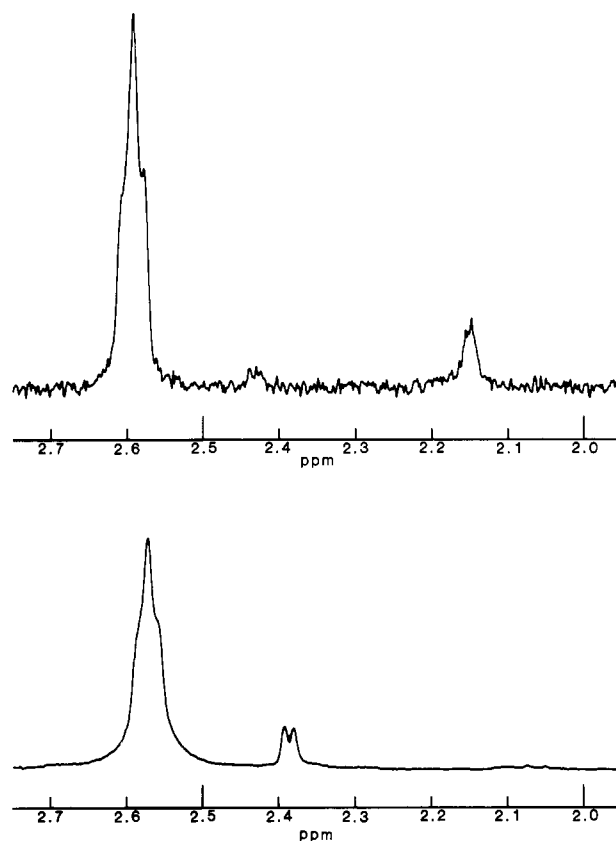


**Figure 3.** FTNMR spectra, 300 MHz of the epoxide region of enzymatically and chemically generated epoxides from 1,1-dideuterio-1-octene. The 300-MHz FTNMR spectrum of the epoxide proton region of (a) the epoxide obtained enzymatically from 1,1-dideuterio-1-octene (top panel) and (b) 1,2-epoxy-1,1-dideuteriooctane obtained by the 1,1-dideuterio-1-octane sample used for the enzymatic epoxidation reaction (bottom panel). (Note the presence of a small amount of undeuterated epoxide due to the undeuterated olefin in the starting material.)

**Table II.**[a] Component Requirements for Product Formation

| assay mixture | epoxide, nmol | aldehyde, nmol |
|---|---|---|
| complete (with NADPH recycling) | 72 | 19 |
| – NADPH recycling | 58 | 8 |
| – rubredoxin | trace | 0 |
| – reductase | trace | 0 |
| – NADPH | <1 | trace |
| boiled monooxygenase | <1 | trace |

[a] The partially purified monooxygenase was assayed as indicated in the Experimental Section using 1,7-octadiene as a substrate and 0.2 mg of partially purified POM. The epoxide produced was 7,8-epoxy-1-octene and the aldehyde was 1-octen-8-al.

From these results it is evident that aldehyde formation from olefins by the partially purified POM proceeds with deuterium migration from carbon 1 to carbon 2. To further establish that aldehyde is an actual product of POM and not a further transformation of the epoxide product, incubations using epoxide as a substrate were performed. As is indicated in Table I, under no condition was aldehyde detected when only epoxide was used as a substrate. Furthermore, Table II shows that both epoxide and aldehyde formation are dependent on the presence of the complete POM system, a further indication that aldehyde is formed during enzymatic turnover. From the time course of product formation shown in Figure 4, it is evident that both aldehyde and epoxide was formed simultaneously, with epoxide being formed about 4 times faster than aldehyde. In addition, formation of both products is similarly dependent on the concentration of POM, again indicating that they are both products of the same system.

**Enzymatic Demethylation.** Formation of aldehydes from an enzymatically produced species along the epoxidation pathway
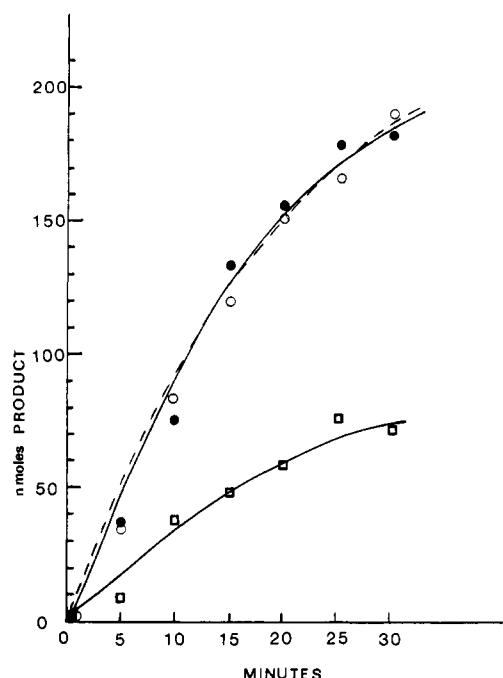
**Figure 4.** Production formation with time using 1,7-octadiene as a substrate. Partially purified *P. oleovorans* monooxygenase was assayed as indicated in the experimental section. A total of 6 mL of assay solution was incubated at 25 °C, and 1-mL of aliquots were removed and extracted every 5 min. ● indicates 7,8-epoxy-1-octene produced without NADPH recycling. O 7,8-epoxy-1-octene, and □ 1-octen-8-al produced with NADPH recycling.

**Table III.**[a] Component Requirements for 1-Heptanol Formation from Heptyl Methyl Ether

| assay mixture | amt of 1-heptanol produced, nmol |
|---|---|
| complete (with NADPH recycling) | 80 |
| − rubredoxin | <3 |
| − reductase | <3 |
| − NADPH | trace |
| − $O_2$[b] | 1.0 |
| boiled monooxygenase | trace |

[a] Assays were performed as indicated in the Experimental Section using heptyl methyl ether as a substrate and 0.2 mg of partially purified POM. [b] Anaerobic conditions were obtained as follows: 0.1 mg of protocatechuate-3,4-dioxygenase were added to a complete assay mixture, which was then flushed in the cold with argon for $^1/_2$ hr. 250 nmol of protocatechuic acid was added, and the mixture was warmed to 25 °C and then incubated at that temperature for 2 min. The assay was initiated by the addition of 2 μL of substrate solution (100 μL of heptyl methyl ether in 1.0 mL of acetone).

implies initial attack of the activated oxygen species on the terminal carbon. Therefore, we examined heptyl methyl ether as a potential substrate for POM, in the anticipation that terminal methyl attack would lead to demethylation.

Incubation of heptyl methyl ether with POM under the usual reaction conditions did indeed result in formation of 1-heptanol, a compound that was readily detected in our standard GC conditions, and in the concurrent formation of formaldehyde as determined by the Nash reaction.[28] Table III shows the component requirements for 1-heptanol formation when heptyl methyl ether was the substrate. It is apparent that the complete POM system is necessary for demethylation to occur and also that molecular oxygen is required.

Support for the conclusion that epoxidation and demethylation both proceed through a common oxygenation pathway is provided by two sets of experiments. First, as shown in Figure 5, epoxidation and demethylation are mutually competitive processes
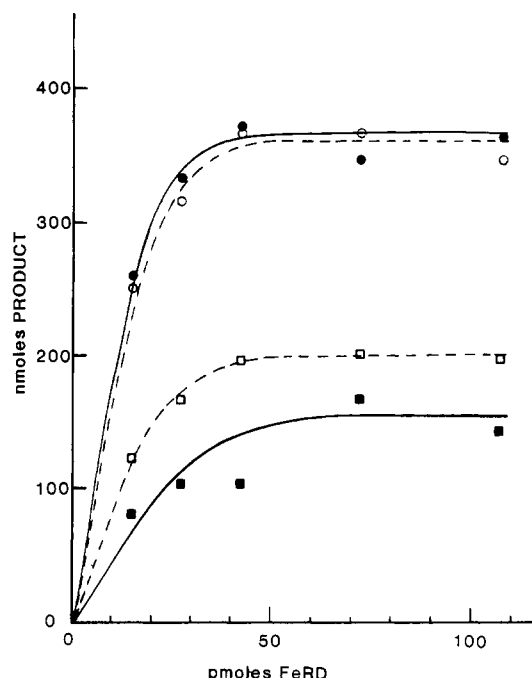
(28) Nash, T. *Biochem. J.* **1953**, *55*, 415–421.



**Figure 5.** Rubredoxin dependencies of epoxidation and demethylation. Assays were performed as in the experimental section. One milligram of POM, various amounts of FeRd, and the NADPH recycling system were used. ● indicates 7,8 epoxy-1-octene produced when 1,7-octadiene was the only substrate, O indicates 1-heptanol produced when heptyl methyl ether was the only substrate, ■ indicates 7,8-epoxy-1-octene, and □ indicates 1-heptanol produced when both 1,7-octadiene and heptyl methyl ether were used as substrates (2 μL of olefin and 1 μL of ether was used for each assay). Aldehyde production from octadiene, which parallels epoxidation and demethylation, is omitted for clarity.

that exhibit parallel rubredoxin dependencies. It is evident from the data that the amounts of both epoxide and heptanol products are considerably diminished when both substrates are present together, despite the fact that rubredoxin, substrates, and NADPH were all present at saturating levels. The second line of evidence is based on our recent finding that acetylenic compounds such as 1,7-octadiyne are potent inhibitors of POM (unpublished observations). This is reminiscent of the well-known suicide inhibition of cytochrome P450 by acetylenes.[29] We find that both epoxidation and demethylation are correspondingly inhibited by 1,7-octadiyne.

## Discussion

The results presented here confirm and extend our previous demonstration that the configuration about the double bond in a simple olefin substrate is inverted upon epoxidation via the non-heme iron POM system. Previously we had utilized whole resting cells of *P. oleovorans* in our experiments with the trans deuterated octadiene substrate, in view or our inability to carry out preparative-scale experiments with the isolated enzyme system. Although we considered possible complications inherent in whole-cell studies, and carried out appropriate control experiments, it was important to confirm this result with isolated enzymes. In all other cases of which we are aware, epoxidation by monooxygenases or closely related model systems maintains the geometric configuration present in the olefin substrate.

Our results clearly show that both the cis- and trans-deuterated olefins undergo the same amount of configurational inversion during the course of enzymatic epoxidation. Furthermore, the epoxide product produced from 1,1-dideuterio-1-octene retains all of the deuterium present in the starting substrate. Taken together these results argue for a simple stepwise epoxidation mechanism which does not involve complex hydrogen abstraction steps and in which the configurational inversion is a consequence

(29) Ortiz de Montellano, P.; Kunze, I. L.; Beilan, H. S.; Wheeler, C. *Biochemistry* **1982**, *21*, 1331–1339.

of the directionalities of initial attack of "activated" oxygen and final closure to the epoxide product. In formulating the details of such a mechanism, our previous demonstration that epoxidation involves stereoselective carbon–oxygen bond formation at carbon 2 must also be taken into account.[30]

Although the formation of chiral epoxides might imply initial attack of "activated" oxygen at carbon 2, our results with heptyl methyl ether suggest that the terminal carbon atom is actually the locus of initial oxygen attack. Our data establish that heptyl methyl ether is very readily demethylated by the POM. Enzymatic demethylation exhibits the characteristics of a reaction proceeding through the normal oxygenation pathway of the POM. All of the enzyme components, as well as NADPH and molecular oxygen, are reported. Demethylation and olefin epoxidation are mutually competitive processes, both are inhibited by acetylenic substrate analogues, and the expected products are formed. Demethylation is also a facile process; under our usual reaction conditions approximately as much heptanol is formed from heptyl methyl ether as epoxide from octadiene. However, we stress that a detailed kinetic study on the formation and lifetimes of the various products in this system has not been carried out.

We favor the view that demethylation proceeds via terminal hydroxylation rather than via initial generation of an oxygen cation radical for several reasons. In the first place, it has long been known that POM exhibits an absolute specificity for terminal hydroxylation of alkanes,[3-6] thus implying that the activated oxygen is impeded from attack at the penultimate atom of a straight chain alkane or ether. It is, of course, conceivable that ether substrates are oriented in a much less restricted way at the active site, which would allow initial electron abstraction from the ether oxygen. However, if this were the case, then partitioning of the initially generated cation radical to form methanol and heptanal might also be expected, and these products are not observed. Finally, recent studies on P-450 dealkylation reactions provide strong precedence for the view that O-dealkylations of anisoles and ethoxycoumarins do not proceed via initial generation of an oxygen cation radical in contrast to the generally accepted mechanism for P-450 catalyzed N- or S-dealkylations.[31,32] Instead, O-dealkylation of such substrates is postulated to proceed via an $\alpha$-hydroxylation mechanism analogous to the one we are proposing here. It should be noted, however, that partitioning between available mechanisms will be affected by the presence of functionalities capable of affecting the stability of cation radical species (e.g., cyclopropyl rings). Thus, at least for our aliphatic ether with POM, both the lack of a radical cation stabilizing substituent and the orientation of the substrate so as to favor terminal attack would disfavor the radical cation pathway.

The present results represent the first demonstration of enzymatic demethylation by the POM system. Microsomes have long been known to catalyze N-, O-, and S-dealkylations, through the involvement of P-450 monooxygenases.[1,33] Bernhardt and co-workers[34] have studied the iron–sulfur protein, putidamonooxin, which catalyzes the O-demethylation of 4-methoxybenzoate. Although no detailed information regarding the ligation environment of the essential iron atom in POM is currently available, it is known that this monooxygenase does not contain acid-labile sulfur[13] and thus is apparently quite distinct from putidamonooxin. Similarly, phenylalanine hydroxylase, which is representative of the mammalian non-heme iron aromatic monooxygenases, has been shown to contain only one essential iron per subunit.[35] It

is thus tempting to predict that the non-heme aromatic monooxygenases such as phenylalanine hydroxylase will also be shown to be capable of catalyzing dealkylation reactions with appropriate substrates.

Further insight into the mechanism of action of the POM, and strong support for the notion of initial attack at the terminal carbon, is provided by our results with the aldehyde products formed from olefin substrates. In our earlier studies with resting cells, these aldehyde products were not detected since they are rapidly metabolized to the corresponding acids. Our more recent preliminary studies with the isolated POM system demonstrated the formation of aldehyde products,[25] and similar observations have recently been reported in the literature for olefin oxygenations by P-450 and model systems.[36,37] Our rationale in examining the aldehyde formed from 1,1-dideuterio-1-octene was the expectation that aldehyde formation via an intramolecular rearrangement of an enzyme-generated species with cationic character at carbon 2 should lead to the presence of deuterium at carbon 2 of the aldehyde product.

Formal chemical precedence for such a process in an intermediate with cationic character is provided by the well-studied pinacol rearrangement of $\alpha$-glycols to aldehydes or ketones[38] where it has been clearly established that 1,2 migration of substituents occurs. It is important to note that for rearrangements of $\alpha$-glycols containing at least one hydrogen substituent, a 1,2-hydrogen shift could conceivably arise via an intermolecular deprotonation pathway, and this possibility received much attention in the early literature on the mechanism of the pinacol rearrangement.[38,39] With deuterated $\alpha$-glycols, such an intermolecular pathway would lead to loss of deuterium in the aldehyde or ketone product. Varying degrees of deuterium loss have been reported for rearrangements of deuterated $\alpha$-glycols, with the results dependent on reaction conditions.[40-42]

It is evident from the mass spectral analysis of the methyl octanoate formed from 1,1-dideuterio-1-octene that deuterium migration from C-1 to C-2 has indeed occurred in the course of enzymatic oxygenation. This represents strong evidence for initial attack of activated oxygen at the terminal carbon atom, followed by intramolecular rearrangement. Integration of the mass spectral data obtained by selective ion counting indicates that about 40% of the methyl octanoate was fully protonted. This could possibly suggest that an intermolecular deprotonation pathway, analogous to that which had been considered in the early literature on the pinacol rearrangement, contributes to the enzymatic pathway. We note, however, that other processes (e.g., exchange in nascent aldehyde prior to trapping, presence of octanoate in POM preparations, etc.) could also contribute to the decreased deuterium content, and, as noted in the Results, extensive loss of deuterium is indeed observed if the aldehyde is not enzymatically converted to the acid in the reaction mixture. In any case, the fact remains that a substantial amount of deuterium migration from C-1 to C-2 has occurred in the course of generation of the aldehyde—but not the epoxide—product from the specifically deuterated olefin, and any mechanism must obviously account for this result.

Taken together, the results reported in this paper and our previous stereochemical and mechanistic studies are consistent with the overall mechanistic view of the POM oxygenation pathway presented in Scheme I. We note at the outset that in this scheme, the putative active-site iron–oxo species is represented as $Fe^V{=}O$ purely as a formalism. No information as to the chemical or electronic nature of such a species is yet available

(30) de Smet, M.-J., et al. (de Smet, M.-J.; Witholt, B.; Wynberg, H. *J. Org. Chem.* **1981**, *46*, 3128) have reported an enantiomeric distribution of 85/15 *R*-(+) epoxide formation from 1-octene. Within the accuracy of the experimental methods this is in agreement with our results with 1,7-octadiene.[16]

(31) Miwa, G. T.; Walsh, J. S.; Lu, A. Y. H. *J. Biol. Chem.* **1984**, *25*, 3000–3004.

(32) Watanabe, Y.; Oae, S.; Iyanagi, T. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 188–195.

(33) Phillips, R. S.; May, S. W. *Enzyme Microb. Techn.* **1981**, *3*, 9–18.

(34) Bernhardt, F. H.; Gersonde, K.; Twilter, H.; Wende, P.; Bill, E.; Trautwein, A. X.; Pfleger, K. In "Oxygenases and Oxygen Metabolism"; Nozaki, M., Yamamoto, S., Ishimura, Y., Coon, J. J., Ernster, L., Estabrook, R. W., Eds.; Academic Press: New York, 1982; pp 63–67.

(35) Gottschall, D. W. Dietrich, R. F.; Benkovic, S. J. Shiman, R. S. *J. Biol. Chem.* **1982**, *257*, 845–849.

(36) Liebler, D. C.; Guengerich, P. *Biochemistry* **1983**, *22*, 5482–5489.

(37) Groves, J. T.; Myers, R. S. *J. Am. Chem. Soc.* **1983**, *5791*–5796.

(38) Collins, C. J. *Quart. Rev. Chem. Soc.* **1960**, *14*, 357–377.

(39) Gould, E. S. In "Mechanism and Structure in Organic Chemistry"; Henry Holt and Co.: New York, 1959; p 602.

(40) Smith, W. B.; Bowman, R. E.; Kmet, T. *J. Am. Chem. Soc.* **1959**, *81*, 997–1003.

(41) Ley, J. B.; Vernon, C. A. *J. Chem. Soc.* **1957**, 2987–2993.

(42) Collins, C. J.; Rainey, W. T.; Smith, W. B.; Kate, I. A. *J. Am. Chem. Soc.* **1959**, *81*, 460–466.

**Scheme I.** Mechanism of POM Oxygenations (Note That the Exact Electronic Distribution within the Various Species Shown and the Electronic State of Iron Are Uncertain. See Text)

EPOXIDATION

ALDEHYDE FORMATION
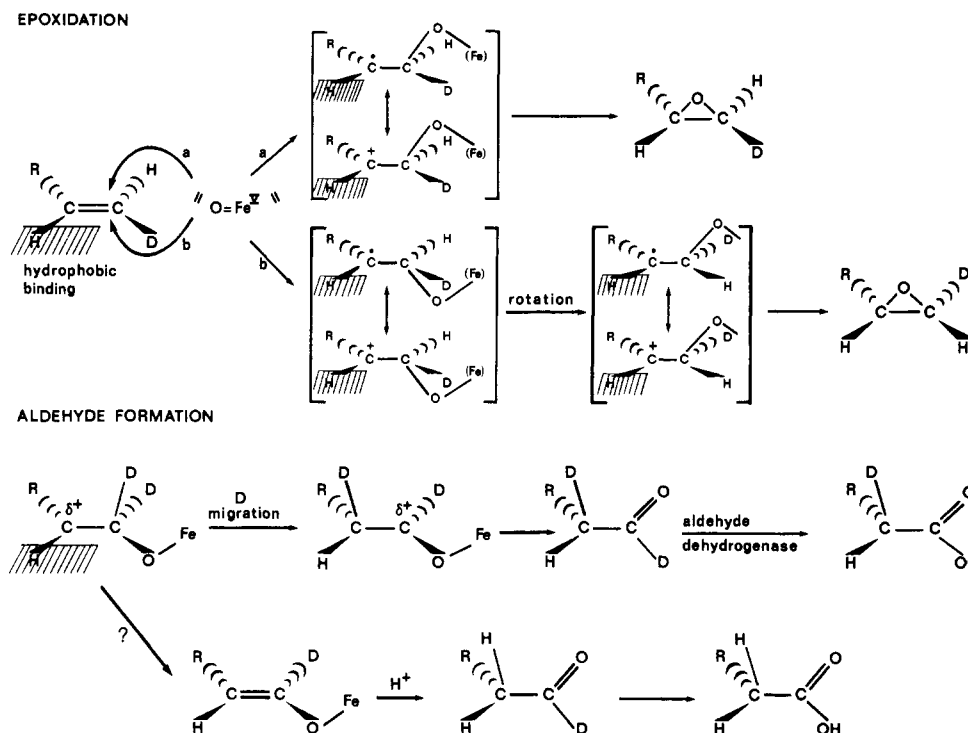
for either POM or other non-heme iron monooxygenases, and mechanisms involving homolytic or heterolytic O–O bond cleavage can be visualized.

Notable aspects of the scheme include the following:

1. Initial oxygen attack occurs only at the terminal carbon, which explains the exclusive formation of only terminal alcohols and terminal epoxides by POM, internal olefins and methylenes being inert to oxygenation. Furthermore, we have never observed the formation of *ketones* from terminal olefins, as would have been expected from initial attack at C-2 followed by the rearrangement shown in the scheme for aldehyde formation. The mechanism of Scheme I obviously also accounts for the O-demethylation activity of POM reported here.

2. Analogous stepwise mechanisms are visualized for epoxidation and hydroxylation with the hydroxylation path being analogous to that proposed by Groves and others,[43-45] for P-450 hydroxylations. Both epoxidation and hydroxylation involve enzyme-generated species with cationic and/or radical character, which collapse to product by ligand transfer from iron.

3. Our stereochemical and configurational studies on epoxidation, as well as Caspi's results on hydroxylation,[46] are fully explained. Initial oxygen attack can abstract any of the methyl hydrogens or can occur from either olefin face at the terminal methylene for hydroxylation and epoxidation, respectively. Our data establish that for epoxidation, initial attack from the *re* face is preferred. In the case of hydroxylation, ligand transfer to complete the hydroxylation process occurs primarily with retention, although some degree of inversion occurs.[46] For epoxidation, however, closure must be from the *si* side of the C-2 due to steric interference of the hydrophobic methylene chain binding region. Thus, the loss of olefin configuration during epoxidation, the complementary extent of geometric retention and inversion for the cis- and trans-deuterated olefins, respectively, and the ste-

reoselectivity in forming *R*-(+) epoxides are all accounted for by Scheme I.

4. Scheme I views aldehyde formation during olefin epoxidation as, in essence, trapping of the enzyme-generated intermediate (or transition state) via rearrangement. The intramolecular migration pathway accounts for our demonstration of substantial intramolecular deuterium migration. It is possible that a deprotonation pathway also contributes to aldehyde formation, but we note once again that deuterium loss via exchange is undoubtedly occurring (see Results). As required by this scheme, our data establish that aldehyde formation indeed exhibits the reaction characteristics expected for a new product arising from a species along the epoxidation pathway.

5. The exact electronic distribution within the intermediate species in Scheme I is not known. Many resonance forms can obviously be visualized with varying degrees of cationic or radical character on C-1 and C-2, and the relative contributions of these, as well as the electron density on iron, cannot be specified. Intermolecular group migration leading to aldehyde should be facilitated by carbocation character in the intermediate species.

Miller and Guengerich[47] have shown that chlorine migration occurs in a transition state formed during P-450 oxidation of trichloroethylene, and they have proposed the involvement of a species analogous to that shown in Scheme I in this process. More recently, Liebler and Guengerich[36] have demonstrated deuterium migration leading to formation of carbonyl compounds during P-450 oxygenation of vinylidene chloride and have suggested a scheme analogous to that proposed here for POM. Thus, the view that non-heme iron and P-450 monooxygenations operate via similar basic mechanisms is supported by the results reported here. We emphasize that the loss of olefinic configuration during epoxidation by POM is unprecedented in P-450 oxygenations, and our confirmation of this finding with complementary results from the cis and trans olefins lends considerable support to the mechanism of Scheme I.

It is important to note that several factors would be expected to affect the partitioning between the pathways shown in Scheme I. The environment at the active site as well as the ligation of the metal would affect the relative stabilities of cationic character

(43) (a) Groves, J. T.; McClusky, G. A. *J. Am. Chem. Soc.* **1976**, *98*, 859–861. (b) Groves, J. T. Van der Puy *J. Am. Chem. Soc.* **1976**, *98*, 5290–5297. (c) Groves, J. T.; Van der Puy *J. Am. Chem. Soc.* **1974**, *96*, 5274–5275.

(44) Groves, J. T.; McClusky, G. A.; White, R. E.; Coon, M. J. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 154–160.

(45) Ortiz de Montellano, P.; Kunze, I. L.; Beilan, H. S.; Wheller, C. *Biochemistry* **1982**, *21*, 1331–1339.

(46) Capsi, E.; Shapiro, S.; Piper, J. V. *Tetrahedron* **1981**, *37*, 3535–3543.

(47) Miller, R. E.; Guengerich, P. F. *Biochemistry* **1982**, *21*, 1090–1097.

*J. Am. Chem. Soc.* 1984, *106*, 7935-7946

7935

at C-1 vs. C-2 and might also control access of a basic species to abstract a proton from C-1. Furthermore, a number of investigators have shown that olefin oxygenation leads to suicide inactivation in P-450 systems,[29] and we have recently reported an olefinic suicide substrate for the copper enzyme dopamine-β-hydroxylase.[48] Thus, although analogous mechanisms may be operative for various monooxygenases, it appears that the distribution of products and the extent, if any, of suicide inactivation reflects differences in the active site and metal ligation environments and the particular electronic nature of the substrate being examined.

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(48) May, S. W.; Mueller, P. W.; Padgette, S. R.; Herman, H. H.; Phillips, R. S. *Biochem. Biophys. Res. Commun.* **1983**, *110*, 161-168.

# General Considerations on Transphosphorylations: Mechanism of the Metal Ion Facilitated Dephosphorylation of Nucleoside 5'-Triphosphates, Including Promotion of ATP Dephosphorylation by Addition of Adenosine 5'-Monophosphate[2]

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**Abstract:** First-order rate constants (50 °C; $I = 0.1$, $NaClO_4$) for the dephosphorylation of uncomplexed nucleoside 5'-triphosphates (= NTP = ATP, GTP, ITP, CTP, UTP, or TTP) are virtually identical at the same pH, reaction occurring by water attack as is evident from the specific rate constants; the reactivity decreases in the phosphate-protonated series $H_2(NTP)^{2-} > H(NTP)^{3-} > NTP^{4-}$. Rate constants are also compared for NTP systems containing metal ions [$M^{2+} = Mg^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$, or $Cd^{2+}$] by combining new results with previous data. The effectiveness of the metal ions to promote ATP dephosphorylation decreases in the order $Cu^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Mn^{2+} > Mg^{2+}$, when the rates at the pH of the maximum promotion are compared. This pH, beginning with $Cu^{2+}$ at pH 6.7, increases within the series $Cu^{2+} < Zn^{2+} < Ni^{2+} < Cd^{2+} < Mn^{2+}$ ($< Mg^{2+}$). The latter order reflects the tendency of these ions to form hydroxo complexes (which occurs in the reverse order) and implies that the nucleophilic attack occurs in an *intra*molecular fashion via an M-OH unit. This view is supported by the calculated specific rate constants and by other experimental results. The sum of all experimental data for the determination of the initial rate of the dephosphorylation ($v_0 = d[PO_4]/dt$) gives evidence that the most reactive species for the pyrimidine-NTP systems has the composition $M_2(R-TP)(OH)^-$, where R-TP represents any triphosphate (including methyltriphosphate) with a noncoordinating terminal organic residue. The most reactive species for the purine-NTP/$Cu^{2+}$ systems at pH <6 has the composition $[M_2(NTP)]_2(OH)^-$; this holds also (including the higher pH range) for the ATP systems with $Ni^{2+}$, $Zn^{2+}$, and $Cd^{2+}$. The most reactive species for $Cu^{2+}$/ATP at pH ≥6.7 is also a dimer with the composition $[Cu(ATP)]_2(OH)^{5-}$. For 1 mM $Cu^{2+}$/NTP 1:1 systems the reactivity decreases in the pH range 2-8 in the order ATP > GTP > ITP > CTP ~ UTP ~ TTP; this can only be explained by the decreasing stacking tendency in this series. An explanation based on the N-7/metal ion interaction, which is crucial for the reactivity of the dimers as shown by NMR experiments, is not applicable because the N-7 coordination tendency follows the order adenosine < inosine < guanosine. The larger dephosphorylation rates of the purine-NTPs compared to the pyrimidine-NTPs in the presence of $M^{2+}$ have their origin in the additional $M^{2+}$/N-7 interaction in purine-NTPs which facilitates the formation of the reactive species. Experiments at pH 5.5 with increasing amounts of $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$, $Ni^{2+}$, $Mg^{2+}$, $Cu(dien)^{2+}$, or $Cu(dpa)^{2+}$ [dien = diethylenetriamine; dpa = di(2-picolyl)amine] added to a 1 mM $Cu^{2+}$/ATP mixture [$Cu(ATP)^{2-}$ is formed to a high degree] indicate that an *inter*molecular attack by water on $[Cu(ATP)(M)]_2$ is also possible if $M^{2+} = Mg^{2+}$, $Ni^{2+}$, $Cd^{2+}$, $Cu(dien)^{2+}$, or $Cu(dpa)^{2+}$. Addition of ligands including several nucleoside 5'-monophosphates (NMP) to a $Cu^{2+}$/ATP system at pH 6.7 supports the view that one purine-NTP in the reactive dimeric species is needed to facilitate transfer of the other into the reactive state. Addition of AMP enhances the reactivity by formation of mixed AMP/ATP stacks, forcing more ATP into the reactive form. However, the "structuring" role of a purine-NTP can only be taken over by a $NMP^{2-}$ having the N-7 and a phosphate group; the effectiveness decreases in the series AMP > GMP > IMP (which is again the order of decreasing stacking tendency). All other ligands (2,2'-bipyridyl, L-tryptophan, phosphate, dien, or dpa) including adenosine, ribose 5'-monophosphate, and tubercidin 5'-monophosphate (= 7-deaza-AMP) inhibit the reaction. The delicacy of the structural arrangement in the stacked dimeric intermediates is evident from addition experiments with adenosine 5'-monophosphate $N(1)$-oxide and 1,$N^6$-ethenoadenosine 5'-monophosphate: both these AMP derivatives have an enhanced base/metal ion coordination tendency (compared with AMP) because they chelate $Cu^{2+}$ to their base, and both are strong inhibitors of the reaction. The proposed structures for the most reactive species have the common feature that a nucleophilic intramolecular attack via a $M^{2+}$-bound $OH^-$ occurs. The connection between the described dephosphorylations in vitro, i.e., trans phosphorylations to $H_2O$, and related reactions in vivo are outlined.

Nucleoside 5'-triphosphates (NTP)[6] play a central role in the metabolism of living cells.[7] They serve as substrates for the enzyme-catalyzed transfers of nucleotidyl or phosphoryl groups—reactions which depend on the presence of divalent metal