

Radical Clock Substrate Probes and Kinetic Isotope Effect Studies of the Hydroxylation of Hydrocarbons by Methane Monooxygenase

Katherine E. Liu,[†] Cathy C. Johnson,[‡] Martin Newcomb,^{*,‡} and Stephen J. Lippard^{*,†}

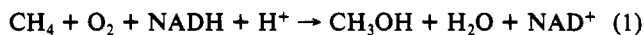
Contribution from the Departments of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and Wayne State University, Detroit, Michigan 48202.

Received August 12, 1992

Abstract: Four mechanistic probes, *trans*-2-phenylmethylcyclopropane (**1**), 2,2-diphenylmethylcyclopropane (**2**), *trans*-1,2-dimethylcyclopropane (**3**), and bicyclo[2.1.0]pentane (**4**), were used as substrates to investigate the possible formation of radical intermediates in the catalytic cycle of the methane monooxygenase (MMO) system from *Methylococcus capsulatus* (Bath) in reactions conducted at 45 °C. Hydroxylation of **1** gave two products in equal amounts, the alcohol *trans*-(2-phenylcyclopropyl)methanol (**1a**) and the phenol *trans*-2-(*p*-hydroxyphenyl)methylcyclopropane (**1c**). The two different products are attributed to discrimination by the enzyme of the two enantiomers of probe **1**, one of which leads to hydroxylation of the aromatic ring and the other to formation of the primary alcohol. The absence of ring-opened product, 1-phenylbut-3-en-1-ol (**1b**), is noteworthy and suggests that the hydroxylation reaction may not proceed through formation of the cyclopropylcarbinyl radical or cation. The former species rearranges to the 3-butenyl radical with a rate constant of $4 \times 10^{11} \text{ s}^{-1}$ at 45 °C, which would require a rebound rate constant for MMO from *M. capsulatus* (Bath) in excess of 10^{13} s^{-1} . The possibility of a stereoelectronic barrier to ring opening in the active site of the enzyme is addressed through a semiquantitative analysis of the rate constant with Marcus theory. Studies with the other radical clock probes support the notion that a significant component of the hydroxylation reaction pathway does not proceed through a radical intermediate. Compound **2**, the cyclopropylcarbinyl radical of which has a ring-opening rate constant of $5 \times 10^{11} \text{ s}^{-1}$, yields exclusively (2,2-diphenylcyclopropyl)methanol (**2a**); **3** ($k = 4.4 \times 10^8 \text{ s}^{-1}$) affords only *trans*-(2-methylcyclopropyl)methanol (**3a**); and **4** ($k = 3 \times 10^9 \text{ s}^{-1}$) gives mainly a mixture of *endo*- and *exo*-2-hydroxybicyclo[2.1.0]pentane (**4a** and **4b**). Kinetic isotope effect experiments were also carried out with derivatives of **1**, mono-, di-, and trideuterated at the methyl position. No intermolecular isotope effect, assessed by comparing the rate of conversion of the undeuterated and trideuterated probes, was observed, a result consistent with C-H bond breaking not being involved in the rate-determining step of the enzymatic reaction. Intramolecular kinetic isotope effects of $k_H/k_D = 5.15$ and 5.03 were obtained from studies of the mono- and dideuterated probes, respectively, which indicates that the hydroxylation reaction involves a substantial C-H bond stretching component. The MMO hydroxylase from *Methylosinus trichosporium* OB3b gave both unrearranged and a small amount (3-5%) of rearranged products with **1**, the ratio of which corresponds to a rebound rate constant of $6-9 \times 10^{12} \text{ s}^{-1}$ at 30 °C. These results are discussed in terms of several possible detailed mechanisms for the MMO hydroxylase reaction.

Introduction

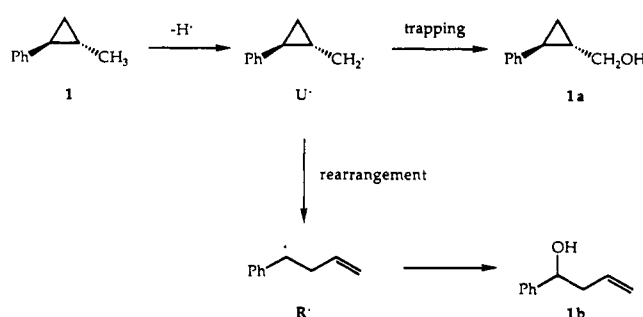
Methane monooxygenase (MMO) is a protein system employed by methanotrophic bacteria to oxidize methane to methanol, a process that provides these organisms with their sole source of carbon and energy (eq 1).¹ The soluble MMOs from *Methy-*



lococcus capsulatus (Bath)² and *Methylosinus trichosporium* OB3b,³ two of the better studied systems, comprise three proteins. Included are a hydroxylase enzyme that binds substrate and dioxygen, a reductase that contains Fe_2S_2 and FAD moieties and accepts electrons from NADH, and a coupling protein that regulates electron transfer between the reductase and the hydroxylase⁴ and also modulates the substrate specificity^{5,6} and redox potentials⁷ of the latter. Recently, much attention has been focused on the hydroxylase enzyme, which is capable of hydroxylating a wide variety of substrates, in addition to methane.^{8,9} EXAFS, EPR, Mössbauer, ENDOR, and other physical studies have shown that the active site in this enzyme contains a dinuclear non-heme iron center in which the metals are probably each coordinated to a histidine and several carboxylate ligands and linked to one another by a monodentate oxygen atom, either from water, hydroxide, or monodentate carboxylate, as well as by a bidentate carboxylate bridge.¹⁰⁻¹² The center resembles that in the crystallographically characterized R2 protein of ribonucleotide reductase.¹³

The catalytic mechanism of the MMO hydroxylase has frequently been compared to that of its widely studied heme analogue, cytochrome P-450.^{8,14-16} In P-450-catalyzed hydroxylations, it is postulated that a high-valent iron oxo, or ferryl, moiety is generated which abstracts a hydrogen atom from the substrate

Scheme 1



to form a hydrocarbon radical that recombines with the coordinated hydroxyl radical before dissociating from the active site.¹⁷⁻²⁰

(1) Anthony, C. *The Biochemistry of Methyloprophs*; Academic Press: New York, 1982, pp 296-379.

(2) Colby, J.; Dalton, H. *Biochem. J.* **1978**, *171*, 461-468.

(3) Fox, B. G.; Surerus, K. K.; Münck, E.; Lipscomb, J. D. *J. Biol. Chem.* **1988**, *263*, 10553-10556.

(4) Green, J.; Dalton, H. *J. Biol. Chem.* **1985**, *260*, 15795-15801.

(5) Froland, W. A.; Andersson, K. K.; Lee, S.-K.; Liu, Y.; Lipscomb, J. D. In *IUCCP Symposium on Applications of Enzyme Biotechnology*; Plenum Press: College Station, TX, 1991; pp 39-54.

(6) Froland, W. A.; Andersson, K. K.; Lee, S.-K.; Liu, Y.; Lipscomb, J. D. *J. Biol. Chem.* **1992**, *267*, 17588-17597.

(7) Liu, K. E.; Lippard, S. J. *J. Biol. Chem.* **1991**, *266*, 12836-12839.

(8) Green, J.; Dalton, H. *J. Biol. Chem.* **1989**, *264*, 17698-17703.

(9) Fox, B. G.; Borneman, J. G.; Wackett, L. P.; Lipscomb, J. D. *Biochemistry* **1990**, *29*, 6419-6427.

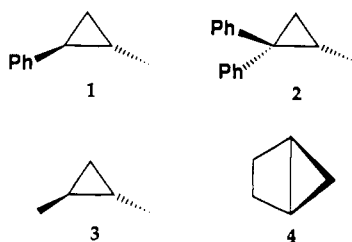
(10) Woodland, M. P.; Patil, D. S.; Cammack, R.; Dalton, H. *Biochim. Biophys. Acta* **1986**, *873*, 237-242.

(11) DeWitt, J. G.; Bentsen, J. G.; Rosenzweig, A. C.; Hedman, B.; Green, J.; Pilkington, S.; Papaefthymiou, G. C.; Dalton, H.; Hodgson, K. O.; Lippard, S. J. *J. Am. Chem. Soc.* **1991**, *113*, 9219-9235.

[†] Massachusetts Institute of Technology.

[‡] Wayne State University.

Chart I



Especially important in establishing the presence of radical intermediates in this mechanism were studies with substrate probes that, following hydrogen atom abstraction to form a radical, isomerize to form, ultimately, a rearranged alcohol product.^{21–23} This process is illustrated for a specific probe, *trans*-2-phenylmethylcyclopropane (1), in Scheme I. Attempts to employ this methodology in studies of the hydroxylation of 1,1-dimethylcyclopropane with the MMO hydroxylase from *M. trichosporium* OB3b were consistent with radical formation, although cationic intermediates were also invoked.¹⁴ Other experimental evidence for the involvement of radical intermediates in the hydroxylation chemistry of MMO from the same organism has been reported.^{8,15,24}

In the present article we describe our investigations of the MMO hydroxylase from both organisms in which four substrate probes, 1–4, illustrated in Chart I, have been used to address the question of radical intermediates and other aspects of the hydroxylation mechanism. Specifically, reactions with *trans*-1,2-dimethylcyclopropane (3) and bicyclo[2.1.0]pentane (4), both of which were used to study the rate constants for rebound with cytochrome P-450,^{21,22} and with two additional, much faster, radical clock substrates, *trans*-2-phenylmethylcyclopropane (1) and 2,2-diphenylmethylcyclopropane (2), were carried out to determine whether substrate radicals are produced during hydroxylation. If, for example, hydrogen atom abstraction from probe 1 at the methyl position were to occur, the resulting cyclopropylcarbinyl radical (U[•], Scheme I) either would be trapped before ring opening to give *trans*-(2-phenylcyclopropyl)methanol (1a) or would ring open to the 1-phenylbut-3-en-1-yl radical (R[•]). Subsequent trapping of radical R[•] would afford 1-phenylbut-3-en-1-ol (1b). Substrate 1 has been employed previously as a hypersensitive radical probe in oxidations by resting cells and crude enzyme preparations of *Pseudomonas oleovorans* monooxygenase.²⁵ In that work, the only oxidation product detected was the ring-opened alcohol, leading to the conclusion that radical intermediates had been

formed and that the oxygen rebound step had a rate constant of $<4 \times 10^9 \text{ s}^{-1}$.

Experimental Section

Isolation and Purification of Proteins. *M. capsulatus* (Bath) was grown as reported previously.^{11,26} The hydroxylase protein was purified and assayed following the described procedure,¹¹ except that Q Sepharose was substituted for DEAE Sepharose in the initial ion exchange chromatography step. The coupling protein (formerly designated protein B) was prepared from an *Escherichia coli* strain harboring a plasmid containing this gene from *M. capsulatus* (Bath)²⁷ and then purified according to published procedures.²⁸ The reductase was purified as described²⁸ except that an HPLC TSKG3000SWG column was employed as a final purification step. Specific activities with propylene were measured at 200–250, 8000–8500, and 4000–4200 milliunits/mg for the hydroxylase, coupling protein, and reductase, respectively.

Growth of *M. trichosporium* OB3b and purification of its hydroxylase were carried out as reported.²⁹ Its specific activity for conversion of propylene to propylene oxide was monitored¹¹ with the use of coupling protein and reductase from *M. capsulatus* (Bath), for which cross-reactivity with the *M. trichosporium* OB3b hydroxylase has been reported previously.^{30,31} The measured activity was 180 milliunits/mg of protein, a value considerably less than expected,²⁹ possibly due to the use of coupling protein and reductase from a different organism. Reactions of 15 μM solutions of the *M. trichosporium* OB3b hydroxylase with 10 mM hydrogen peroxide produced 0.19 mM propylene oxide in a 3-min reaction, however, which compares favorably with literature reports that 44 μM hydroxylase and 10 mM H_2O_2 yielded approximately 0.4 mM propylene oxide in the same time period.¹⁶ The measured iron content, 2.2 mol of Fe/mol of hydroxylase, was less than that found by others, 4.3 mol of Fe/mol of protein,²⁹ for which we have no obvious explanation.

Synthesis and Characterization of Substrates and Products. The substrate *trans*-2-phenylmethylcyclopropane (1) was prepared as previously reported³² by reducing commercially available *trans*-2-phenylcyclopropanecarboxylic acid or its ethyl ester to alcohol 1a, *trans*-(2-phenylcyclopropyl)methanol, with LiAlH_4 , converting the alcohol to the mesylate, and reducing the mesylate with LiEt_3BH . As an alternative, conversion of alcohol 1a to the corresponding chloride by treatment³³ with PCl_5 and CCl_4 followed by reduction with LiAlH_4 gave hydrocarbon 1 and ring-opened product 4-phenylbut-1-ene; treatment of the mixture with *m*-chloroperoxybenzoic acid to epoxidize the alkene byproduct, followed by silica gel chromatography, gave pure 1. Deuterated analogues of 1 were prepared by the mesylate route with LiAlD_4 as the source of deuterium. Their NMR and mass spectra were consistent with the expected amount of deuterium incorporation for each analogue.

The substrate 2,2-diphenylmethylcyclopropane (2) was prepared by a sequence similar to that described above for 1. Reaction³⁴ of ethyl diazoacetate in the presence of CuSO_4 with 1,1-diphenylethene gave ethyl 2,2-diphenylcyclopropanecarboxylate, which was reduced with LiAlH_4 to (2,2-diphenylcyclopropyl)methanol (2a). Conversion of 2a to the mesylate followed by LiEt_3BH reduction gave 2.

Authentic samples of the products obtained by the MMO-catalyzed oxidation of substrates 1 and 2 were prepared in the following manner. *trans*-(2-Phenylcyclopropyl)methanol (1a) and its dideuterated analogue were obtained from the synthesis of 1, and alcohol 2a was available in the preparation of 2, as indicated above. Monodeuterated 1a was prepared by LiAlD_4 reduction of *trans*-(2-phenylcyclopropyl)methanol which, in turn, was obtained from the acid by sequential treatment with H_2BSMe_2 and pyridinium chlorochromate.³⁵ 1-Phenylbut-3-en-1-ol (1b) and 1,1-diphenylbut-3-en-1-ol (2b) were prepared by reactions of allylmagnesium chloride with benzaldehyde and benzophenone, respectively.

An authentic sample of *trans*-2-(*p*-hydroxyphenyl)methylcyclopropane (1c) was prepared by the following sequence. Reaction³⁴ of *p*-vinylanisole with ethyl diazoacetate gave a mixture of ethyl *cis*- and *trans*-2-(*p*-methoxyphenyl)cyclopropanecarboxylates, which was separated by column chromatography and crystallization. The *trans* isomer, identified

(12) Hendrich, M. P.; Fox, B. G.; Andersson, K. K.; Debrunner, P. G.; Lipscomb, J. D. *J. Biol. Chem.* **1992**, *267*, 261–269.

(13) Nordlund, P.; Sjöberg, B.-M.; Eklund, H. *Nature* **1990**, *345*, 593–598.

(14) Ruzicka, F.; Huang, D.-S.; Donnelly, M. I.; Frey, P. A. *Biochemistry* **1990**, *29*, 1696–1700.

(15) Rataj, M. J.; Kauth, J. E.; Donnelly, M. I. *J. Biol. Chem.* **1991**, *266*, 18684–18690.

(16) Andersson, K. K.; Froland, W. A.; Lee, S.-K.; Lipscomb, J. D. *New J. Chem.* **1991**, *15*, 411–415.

(17) McMurry, T. J.; Groves, J. T. In *Cytochrome P-450 Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum Publishing Corp.: New York, 1986; pp 1–28.

(18) Ortiz de Montellano, P. R. In *Cytochrome P-450 Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum Publishing Corp.: New York, 1986; pp 217–271.

(19) Mansuy, D.; Battioni, P. In *Activation and Functionalization of Alkanes*; Hill, C. L., Ed.; Wiley: New York, 1989; Chapter VI.

(20) Guengerich, F. P. In *Biological Oxidation Systems*; Reddy, C. C., Hamilton, G. A., Madyastha, K. M., Eds.; Academic Press: San Diego, 1990; Vol. 1, pp 51–67.

(21) Ortiz de Montellano, P. R.; Stearns, R. A. *J. Am. Chem. Soc.* **1987**, *109*, 3415–3420.

(22) Bowry, V. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1991**, *113*, 5699–5707.

(23) Bowry, V. W.; Luszyk, J.; Ingold, K. U. *J. Am. Chem. Soc.* **1991**, *113*, 5687–5698.

(24) Deighton, N.; Podmore, I. D.; Symons, M. C. R.; Wilkins, P. C.; Dalton, H. J. *Chem. Soc., Chem. Commun.* **1991**, 1086–1088.

(25) Fu, H.; Newcomb, M.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 5878–5880.

(26) Woodland, M. P.; Dalton, H. J. *Biol. Chem.* **1984**, *259*, 53–59.

(27) Wu, W. W.; Rosenzweig, A. C.; Lippard, S. J. Unpublished results.

(28) Pilkington, S. J.; Dalton, H. In *Methods In Enzymology*; Academic Press: New York, 1990; Vol. 188, pp 181–190.

(29) Fox, B. G.; Froland, W. A.; Jollie, D. R.; Lipscomb, J. D. In *Methods In Enzymology*; Academic Press: New York, 1990; Vol. 188, pp 191–202.

(30) Stirling, D. I.; Dalton, H. *Eur. J. Biochem.* **1979**, *96*, 205–212.

(31) Dalton, H.; Higgins, I. J. *Antonie van Leeuwenhoek* **1987**, *53*, 23–28.

(32) Gajewski, J. J.; Squicciarini, M. P. *J. Am. Chem. Soc.* **1989**, *111*, 6717–6728.

(33) Falorni, M.; Lardicci, L.; Giacomelli, G. *J. Org. Chem.* **1986**, *51*, 5291–5294.

(34) Blatchford, J. K.; Orchin, M. *J. Org. Chem.* **1964**, *29*, 839–843.

(35) Brown, H. C.; Kulkarni, S. U.; Rao, C. G. *Synthesis* **1979**, 704–705.

by NMR spectroscopy, was reduced with LiAlH_4 , and the resulting alcohol was converted to its mesylate, which was then reduced with LiEt_3BH . The resulting *trans*-2-(*p*-methoxyphenyl)methylcyclopropane was demethylated³⁶ with NaSEt in DMF to give the desired product, which was purified by preparative GC on a Carbowax 20M column. Authentic samples of the possible aromatic hydroxylated products from **2** were not prepared; no components consistent with these products were observed by GC mass spectral analysis of the oxidation mixtures.

Full details of the above synthetic procedures are provided as supplementary material.

trans-1,2-Dimethylcyclopropane (**3**), *trans*-(2-methylcyclopropyl)methanol (**3a**), 4-penten-2-ol (**3b**), and 2-methylbut-3-en-1-ol (**3c**) were obtained from Wiley Organics (Coschocton, OH). Bicyclo[2.1.0]pentane (**4**) was synthesized according to a literature method.³⁷

Enzymatic Reactions. Reactions with MMO from *M. capsulatus* (Bath) were carried out at 45 °C with the reconstituted enzyme system in the following manner. Solutions were prepared that contained 1.2 mg (10 μM) of hydroxylase, 0.2 mg (24 μM) of coupling protein, and 0.4 mg (20 μM) of reductase, all in 25 mM MOPS [MOPS = 3-(*N*-morpholino)propanesulfonic acid], pH 7.0, in a final volume of 0.5 mL. A 0.5- μL portion of substrate was then added by means of a microsyringe to achieve a maximum concentration of ~ 8 mM, most of the substrates not being completely soluble prior to conversion to products. The reaction mixture was placed in a 10-mL glass vial and sealed with a Suba-Seal septum. After addition of a 25- μL portion of a 0.1 M stock solution of ethanol-free NADH to initiate the hydroxylation reaction, the vial was placed in a shaker-incubator maintained at 45 °C. Control reactions were performed in an identical manner except that no NADH was added. Reactions were allowed to proceed for 15 min, a time sufficient to afford at least 30 mol of product/mol of enzyme in those cases where authentic standards were available.

Studies with the hydroxylase from *M. trichosporium* OB3b and substrate **1** were carried out at 30 °C in one of two ways. Coupling protein and reductase from *M. capsulatus* (Bath) were incubated with the hydroxylase in the presence of NADH. The protein and NADH concentrations were the same as described in the preceding paragraph. Alternatively, reactions were performed by using 10 mM hydrogen peroxide and *M. trichosporium* OB3b hydroxylase only, a known shunt for this enzyme.¹⁶ Substrate was added to the reaction mixture, which was then incubated as described above. Again, at least 30 turnovers were completed before the reaction was terminated.

Instrumentation. Products from enzyme oxidations were analyzed with a Hewlett-Packard (HP) Model 5890 gas chromatograph either equipped with an FID detector or interfaced to an HP Model 5971A mass spectrometer (EI, 70 eV). For GC and GC/MS analysis, either a cross-linked FFAP (HP) or a cross-linked methyl silicon (HP) capillary column was employed, depending on which column gave better resolution of the reaction components. The column dimensions were 10 m \times 0.5 mm \times 1.0 μm for GC analysis and 25 m \times 0.2 mm \times 0.33 μm for GC/MS analysis. Slightly different instrumental conditions were used to analyze the different reactions, as indicated in Table S1 (supplementary material).

Product Identification and Quantitation. Products were isolated by thorough extraction of the reaction mixture with diethyl ether or ethyl acetate. The septum of the reaction vial was removed, 5 mL of extracting solvent was added, and the septum was immediately replaced. The mixture was incubated for at least 10 min, the organic layer was then removed, and the entire procedure was repeated twice. The organic layers were combined, dried over anhydrous K_2CO_3 , and then concentrated by slow evaporation under Ar to a volume of ~ 100 μL . After the addition of an appropriate internal standard (vide infra), a 0.2–0.5- μL sample of concentrated extract was injected into the gas chromatograph. The retention times and mass spectra of the products were compared to those of authentic standards.

Components of the hydroxylation reaction mixture for **1**–**3** were quantitated by constructing standard curves in the following manner. Solutions containing known concentrations of a given component and a constant amount of an internal standard dissolved in ethyl acetate or diethyl ether were injected into the GC. The ratio of the peak area corresponding to the component with that of the standard was then plotted against the concentration of added component in the sample. A linear plot was obtained and subsequently used as a calibration curve to determine the concentration of the component in extracts obtained from reactions with the enzyme. Plots were constructed in this manner for alcohols **1a**–**3a**, rearranged alcohols **1b**–**3b** and **3c**, 2-(*p*-hydroxyphenyl)methylcyclopropane (**1c**), and substrates **1**–**3**. Since no **1b** was detected in reactions of MMO from *M. capsulatus* (Bath) with **1** (vide

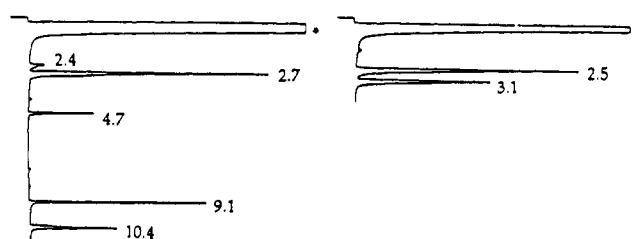


Figure 1. Typical GC traces from extracts following hydroxylation with MMO from *M. capsulatus* (Bath) of **1**, left, and **3**, right. The peaks at 4.7 (left) and 2.5 min (right) are due to the internal standards methyl benzoate and (1-methylcyclopropyl)methanol, respectively. The asterisks designate the solvent fronts.

infra), mass balance determinations were made to ensure that the amount of unreacted substrate and products corresponded to the amount of substrate added to the reaction. In addition, reactions containing 0.8 mM **1b** as well as the usual quantity (8 mM) of **1** were carried out to determine if the latter would survive the hydroxylation conditions and/or compete with **1** for the enzyme. Mock experiments were carried out without addition of NADH, and the substrate was recovered following the usual extraction and concentration procedures.

Isotope Effect Studies. Reactions with MMO from *M. capsulatus* (Bath) were investigated with **1** and its mono-, di-, and trideuterated derivatives at the methyl position. An intramolecular isotope effect was investigated through studies of the mono- and dideuterated derivatives. The product alcohol **1a** from reactions with each substrate was analyzed by GC/MS. The isotope effect was then calculated by comparison of the intensities, after statistical corrections, at m/z 148 and 149 for alcohols arising from the monodeuterated substrate and at m/z 149 and 150 for alcohols derived from the dideuterated substrate.

Intermolecular isotope effects were evaluated by comparison of the rate of formation of **1a** in MMO-catalyzed reactions with the undeuterated and the trideuterated derivatives. The experiment was performed in two ways, as a competitive reaction and as separate reactions with each substrate. Competition experiments were performed with equal amounts (0.5 μL) of both derivatives present in the reaction vial. The mass spectral intensity at m/z 148, representing reaction of the undeuterated substrate, was divided by the intensity at m/z 150, representing reaction with the trideuterated substrate, and the ratio was taken as the intermolecular isotope effect. In separate reactions with the two derivatives, 2-(*p*-hydroxyphenyl)methylcyclopropane (**1c**), a product of the enzymatic reaction (vide infra), was used as an internal standard for the purpose of quantitation. The amount of **1a** relative to **1c** was calculated for each derivative. The ratio of alcohol to phenol for the undeuterated derivative was divided by the same ratio for the trideuterated derivative to provide the intermolecular isotope effect.

Results

Table I reports the results of the catalytic hydroxylation of substrates **1**–**4** by MMO from *M. capsulatus* (Bath). For substrates **1**–**4**, the ratio of experimentally observed alcohols, corresponding to hydroxylation without and with rearrangement, is indicated. In addition, the table includes the known rearrangement rates of the appropriate radical intermediates. Chromatographic retention times are provided in Table II, and typical GC traces for components of the reaction mixtures are shown in Figure 1.


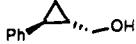

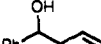

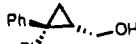
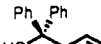

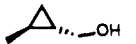
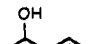
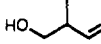



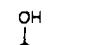
The hydroxylation of **1** yielded two products as revealed by both GC and GC/MS analysis. They were identified as alcohol **1a** and 2-(*p*-hydroxyphenyl)methylcyclopropane (**1c**)³⁸ by comparison of their retention times and mass spectra with those of authentic standards. Quantitation of the peaks revealed that the two products are formed in approximately equal concentrations and at equal rates over a 20-min reaction period (Figure S1, supplementary material). To ensure that all added material was accounted for in the analysis, mass balance studies were carried out. The amount of material recovered in control reactions run in the absence of reductant corresponded to $84.4 \pm 0.6\%$ of the substrate initially added, the remainder apparently being lost

(36) Feutrill, G. I.; Mirrington, R. N. *Tetrahedron Lett.* **1970**, 1327–1328.

(37) Gassman, P. G.; Mansfield, K. T.; Taylor, G. N.; Wiberg, K. B. *Org. Syn.* **1969**, *49*, 1–6.

(38) It is possible that the ortho and meta isomers of **1c** coeluted with **1c** in the GC analysis and were thus not detected. Separation of enzymatic reaction mixtures with **1** by HPLC (C18 reverse-phase) yielded only three peaks, which corresponded to **1**, **1a**, and **1c**. It is unlikely that the ortho and meta isomers of **1c** would coelute with **1c** in both GC and HPLC analysis.

Table I. Expected Products and Observed Ratios for Reactions with MMO from *M. capsulatus* (Bath)

substrate	unrearranged product(s)	rearranged alcohol(s) expected	obsd ratio of unrearranged to rearranged alcohol	rate constant for radical rearrangement, s ⁻¹ , at 45 °C
	 		>100:1	4 × 10 ¹¹ ref 42
			>25:1	5 × 10 ¹¹ ref 42
		 	both >100:1	3b: 2.0 × 10 ⁸ 3c: 2.4 × 10 ⁸ ref 23
	 		80:1 ^a	3 × 10 ⁹ refs 23, 40

^a See text.**Table II.** Retention Times of Reactants and Products in the Reaction of 1–4 with the MMO Hydroxylase^a

compd	time (min)	compd	time (min)	compd	time (min)
1	2.7 (2.4) ^b	2a	9.5	3c	2.3
1a	9.1	2b	6.5	4	2.0
1b	7.1	3	0.7	4a	6.5
1c	10.4	3a	3.1	4b	8.2
2	3.1	3b	1.5	4c	7.3

^a Reported times for reactions with 1–3 are for GC analyses, whereas times for reactions with 4 are for GC/MS analyses. ^b This peak forms upon thermal rearrangement of the probe in the gas chromatograph.

through the various extraction manipulations. When NADH was present to initiate and sustain enzyme turnover, 85.0 ± 0.5% of the material added was recovered, 5.0 ± 0.2% of which was **1a**, and 5.1 ± 0.1%, **1c**. No rearranged alcohol **1b** was detected. The possibility that **1b** formed but was unstable under the hydroxylation conditions was investigated through analysis of a reaction containing 8 mM **1** and 0.8 mM **1b**. In the presence of NADH, 78.6 ± 1.3% of **1b** was recovered, compared to 78.2 ± 1.0% in control reactions without NADH. This result indicates that **1b** is unaffected during the hydroxylation reaction. The yields of **1a** and **1c** remained unchanged.

Hydroxylation studies of the mono- and dideuterated derivatives of substrate **1** gave values for k_H/k_D of 5.15 ± 0.38 and 5.03 ± 0.42, respectively, for the intramolecular isotope effect. The intermolecular isotope effect was determined by comparing results for **1** with those for its trideuterated derivative. A value of 1.18 ± 0.15 was obtained for reactions that were performed separately, whereas competitive reactions performed with both derivatives present in the reaction mixture yielded a value of 0.89 ± 0.10 (see Experimental Section). In the competition study, the ratio of phenols (**1c-d₃**:**1c-d₁**) was 1.1.

GC analysis of hydroxylation reactions with **2** revealed only a single product, which was identified by comparison to an authentic standard as the alcohol **2a**. No peaks that might arise from rearranged alcohol **2b** or aromatic hydroxylation products were detected. A plot showing the rate of product formation is given in Figure S2.

Analysis of the extracts from reactions with *trans*-1,2-dimethylcyclopropane (**3**) revealed only one product, the concentration of which increased linearly with time over a 10-min period (Figure S1). It was identified as the unrearranged alcohol, *trans*-(2-methylcyclopropyl)methanol (**3a**), through comparison of its GC retention time with that of an authentic standard. No 4-penten-2-ol (**3b**) or 2-methylbut-3-en-1-ol (**3c**) was detected.

The substrate bicyclo[2.1.0]pentane (**4**) afforded three products upon reaction with MMO (Figure S3), each of which had its parent ion at m/z 84 as determined by GC/MS analysis. No authentic standards were available for the expected products, but one product could be positively identified as *endo*-2-hydroxybicyclo[2.1.0]pentane (**4a**) by comparison of its mass spectrum with one published previously.²¹ The second major peak in the gas chromatogram was assigned as *exo*-2-hydroxybicyclo[2.1.0]pentane (**4b**). Unfortunately no mass spectral data for this compound were available for comparison. The peak had approximately the same intensity (~85%) in the total-ion chromatogram of the reaction extracts as that of **4a**. Its mass spectrum differed from those of **4a** and 3-cyclopenten-1-ol (**4c**), for which mass spectral data are also available.³⁹ Hydroxylation of **4** by cytochrome P-450 yielded only **4a** and **4c**.^{21,22} The failure of this enzyme to afford **4b** was attributed to constraints at the active site. It may be that the active site of MMO is less discriminating, which could account for the formation of both **4a** and **4b**.

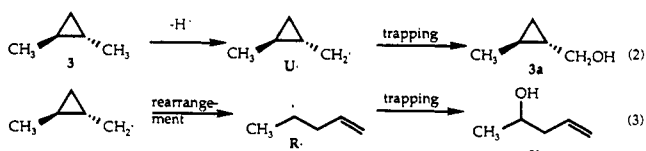
The third product from reactions with **4** was present in only trace amounts and was assigned as 3-cyclopenten-1-ol (**4c**). It has been reported previously that **4a** undergoes thermal rearrangement to **4c**.^{21,22} In particular, when **4a** isolated from the hydroxylation of **4** by MMO was injected into the GC/MS, the ratio of **4c** to **4a** was found to be ~1:30. Compound **4b** is expected to behave similarly. The intensity of the peak corresponding to **4c** was also found to increase relative to those for **4a** and **4b** when the reactions were run at 45 °C for longer times (Figure S3). Increasing the injector and oven temperatures of the gas chromatograph produced a similar effect. The ratio of **4c** to **4a** was 1:40, or including both **4a** and **4b**, 1:80, and increased with longer

incubation times at 45 °C to about 1:15. It is therefore likely that a significant amount, if not all, of **4c** obtained in these experiments is due to thermal rearrangement of **4a** and **4b**. The amount of **4c** present in the enzymatic reactions is small enough that its presence could be solely the result of thermal rearrangement of **4a** and **4b**.²¹

Experiments were also carried out with **1** and the hydroxylase from *M. trichosporium* OB3b. In contrast to reactions with MMO from *M. capsulatus* (Bath), three products were observed. They were identified as unrearranged alcohol **1a**, 2-(*p*-hydroxyphenyl)methylcyclopropane (**1c**), and rearranged product **1b**. The ratio of unrearranged to rearranged alcohol was found to be 32:1 in reactions performed with coupling protein and reductase from *M. capsulatus* (Bath) and 22:1 for reactions using hydrogen peroxide.

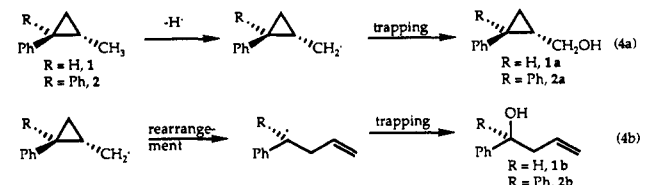
Discussion

Analysis of Products Formed. In this work, four hydrocarbon substrates were employed as mechanistic probes in oxidations catalyzed by the MMO of *M. capsulatus* (Bath). In each case, if a hydrogen atom were abstracted from the probe, the resulting radical could either rebound to form the unrearranged product or ring open to afford the rearranged product (Scheme 1). In no case was rearrangement observed. For *trans*-1,2-dimethylcyclopropane (**3**) only a single product formed, corresponding to the unrearranged alcohol (**3a**, eq 2). No allylic alcohol was detected



(**3b**, eq 3), implying that no more than 1% of this product formed during the hydroxylation reaction. From the known rate constant for rearrangement of the corresponding cyclopropylcarbinyl radical (Table I),²³ and assuming that this rate constant is the same for substrate free in solution and bound to the enzyme (vide infra), we estimate that the rebound rate constant of a radical intermediate would have to be at least $4 \times 10^{10} \text{ s}^{-1}$ at 45 °C. A similar analysis^{23,40-42} of the results for reactions with compounds **4**, **1**, and **2** raises the rebound rate constant limits to 3×10^{11} , 4×10^{13} , and $1 \times 10^{13} \text{ s}^{-1}$, respectively. The latter two values exceed the rate constant of decomposition of a transition state and are incompatible with a discrete radical intermediate.

The rate constants for ring opening of radicals derived from **1** and **2** (eq 4a,b) have been measured indirectly by competition against PhSeH trapping.^{41,42} At room temperature, the ring



openings have rate constants of $3 \times 10^{11} \text{ s}^{-1}$ and $5 \times 10^{11} \text{ s}^{-1}$, respectively, which places these reactions among the fastest reported radical rearrangements involving a bond-breaking step. With such a velocity, detectable amounts of ring-opened products are expected to be produced from these radicals even when the fastest possible trapping reactions compete. Therefore, the absence of ring-opened alcohols **1b** and **2b** suggests that probes **1** and **2** were converted directly to alcohols **1a** and **2a** without formation of radicals. On the other hand, a small amount of rearranged product **1b** was observed in the studies of MMO from *M. tri-*

chosporium OB3b. From the ratio of unrearranged to rearranged alcohol the rate constant for rebound was calculated to be $6\text{--}10 \times 10^{12} \text{ s}^{-1}$. The observed differences in the results for MMO from the two different sources will be discussed later.

In the case of the chiral substrate probe **1**, oxidation occurred not only at the methyl group but also at the phenyl ring to form 2-(*p*-hydroxyphenyl)methylcyclopropane (**1c**) (Table I).³⁸ The fact that **1a** and **1c** are produced in equal quantities over the course of the reaction suggested that the two products might arise from different enantiomers of the probe. Preliminary studies with partially resolved **1** tend to confirm this possibility (unpublished results), but more data are required for the hydroxylation of both pure enantiomers before a definitive statement can be made.

Deuterium Isotope Effects. Studies of the reaction of deuterated derivatives of **1** with MMO from *M. capsulatus* (Bath) failed to reveal any intermolecular isotope effect. Both individual and competitive reactions with the undeuterated and trideuterated derivatives yielded values of $(k_H/k_D)_{\text{obsd}}$ of approximately 1.0 and in good agreement with one another, considering the error limits. It is important to note that the ratio of undeuterated and trideuterated phenols observed in the competition experiment was 1.1. When combined with the ratio of 0.89 for the undeuterated and dideuterated alcohols **1a**, this result shows that the overall rates of oxidation of the undeuterated and trideuterated substrates were equal. Thus, there was essentially no kinetic discrimination for substrates by the enzyme, and this behavior suggests that C–H bond breaking is not rate-limiting in the overall hydroxylation reaction. Such a result is consistent with some,^{15,43} but not all,⁴⁴ previous studies of MMO hydroxylation reactions using deuterated substrates.

Given that the overall oxidation rates of **1-d₀** and **1-d₃** were the same, the product distributions should be considered in more detail. If oxidation of each of the enantiomers of substrate **1** could have occurred at either the methyl group or the aromatic ring, and if rotation can occur in the active site, then a kinetic isotope effect should have been observed in the oxidations of the undeuterated and trideuterated probes, with substantially more phenol product being formed in the latter case. This result is clearly predicted from the intramolecular kinetic isotope effects that were found (vide infra). That such was not the case reinforces our speculation that products **1a** and **1c** arise mainly from different enantiomers of the probe.

In contrast to the lack of an intermolecular isotope effect, a significant intramolecular isotope effect of approximately 5.1 (statistically corrected) was observed in oxidations of both the mono- and dideuterated probes **1**. In each case, the observed isotope effects are equal to the primary kinetic isotope effects (k_H/k_D) divided by a secondary kinetic isotope effect that results from the fact that in both cases an additional deuterium atom remains bound after oxidation of a C–H bond. The data do not permit an extraction of the secondary kinetic isotope effect, but it is clear that the $(k_H/k_D)_{\text{obsd}}$ ratio signals a substantial C–H bond stretching component in the transition state of the rate-limiting step in the oxidation reaction. The value of $(k_H/k_D)_{\text{obsd}} = 5.1$ may be compared with the intramolecular isotope effect of 7.8 observed in the oxidation of probe **1** with *P. oleovorans* monooxygenase.²⁵ Recently, a value of $k_H/k_D = 4.2 \pm 0.2$ was reported for MMO from *M. trichosporium* OB3b with the substrates (*S*)- and (*R*)-[1-²H,1-³H]ethane, in good agreement with the present results.⁴⁵ Cytochrome P-450 dependent enzymes oxidize a number of substrates with intramolecular isotope effects of 7–14.²² Intramolecular isotope effects for hydrogen atom abstractions by the reactive *tert*-butoxy radical studied at temperatures in the vicinity of 25 °C range from 1.2 to 5.4, and isotope effects for hydrogen abstractions by less reactive peroxy radicals are greater.⁴⁶

(40) Newcomb, M.; Manek, M. B.; Glenn, A. G. *J. Am. Chem. Soc.* **1991**, *113*, 949–958.

(41) Newcomb, M.; Manek, M. B. *J. Am. Chem. Soc.* **1990**, *112*, 9662–9663.

(42) Newcomb, M.; Johnson, C. C.; Manek, M. B.; Varick, T. R. *J. Am. Chem. Soc.*, in press.

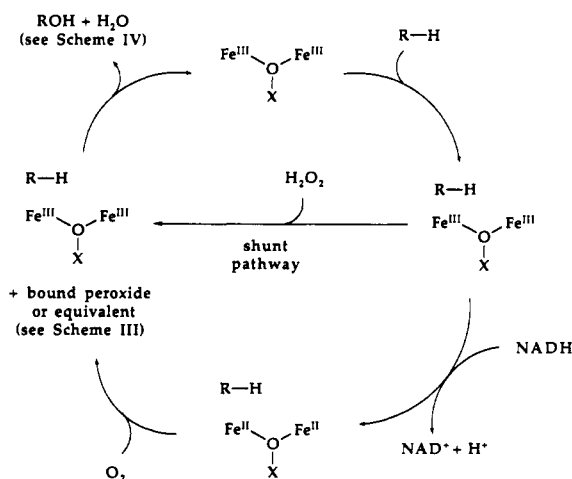
(43) Shimoda, M.; Okura, I. *J. Mol. Catal.* **1992**, *72*, 263–267.

(44) Green, J.; Dalton, H. *Biochem. J.* **1989**, *259*, 167–172.

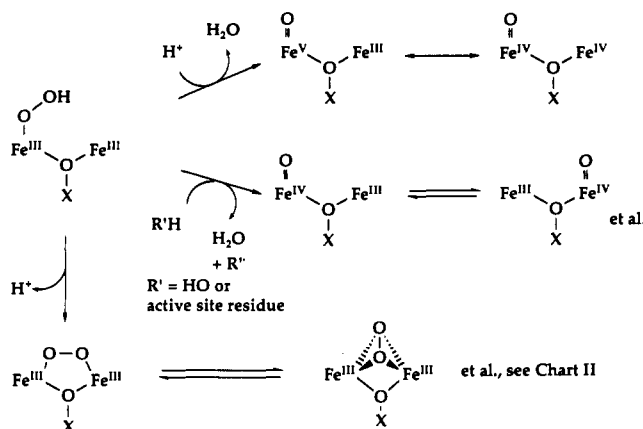
(45) Priestley, N. D.; Floss, H. G.; Froland, W. A.; Lipscomb, J. D.; Williams, P. G.; Morimoto, H. *J. Am. Chem. Soc.* **1992**, *114*, 7561–7562.

(46) Howard, J. A.; Scaiano, J. C. *Landolt-Börnstein Numerical Data and Functional Relationships in Science and Technology*; Springer-Verlag: Berlin, 1984; Vol. 13, Subvol. d, pp 125–127.

Scheme II

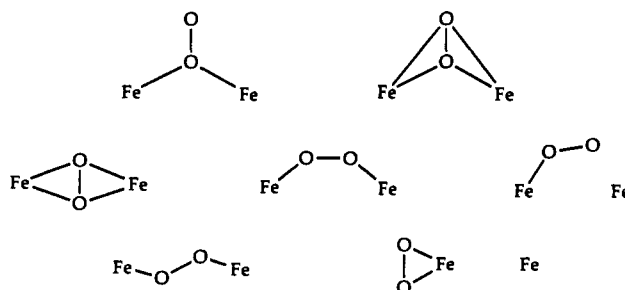


Scheme III



Mechanistic Considerations: Formation of the Dioxygen-Activated Diiron Center. Scheme II outlines a reasonable working hypothesis for the catalytic mechanism of MMO. Substrate first binds to the complete system, which contains all three protein components. Addition of NADH then effects a two-electron reduction of the hydroxylase from the oxidized Fe^{III}Fe^{III} to the fully reduced Fe^{II}Fe^{II} form, bypassing the inactive⁴⁷ Fe^{II}Fe^{III} state.^{7,44} The fully reduced hydroxylase next reacts with dioxygen in a two-electron step to form a diiron(III) peroxide complex which, either itself or upon transformation via two more electron transfer steps at the diiron center, is sufficiently activated to attack the hydrocarbon substrate. The nature of this transformed species, if it exists, is currently unknown, but it could involve a high-valent iron oxo moiety (Scheme III), by analogy with that proposed for cytochrome P-450.¹⁷ The second iron atom in the MMO hydroxylase active site would stabilize such a unit through redox charge delocalization, as shown in Scheme III, in much the same fashion that cytochrome P-450 is thought to facilitate the formation of a high-valent iron oxo species through oxidation of the porphyrin ring to a π -cation radical. Alternatively, the internal two-electron transfer step could produce an active site radical (R[•]), such as the hydroxyl radical or a ligand-based radical involving an amino acid residue, and an iron(IV) center that would again be stabilized by tautomerization and electron transfer involving the other iron atom (Scheme III). Although there is presently no direct evidence for such a radical species in the MMO hydroxylase active site, the combination of a redox-active metal with a protein-derived radical to effect oxygen-activated redox transformations is known in the related iron enzyme ribonucleotide reductase,⁴⁸ in the heme peroxidase prostaglandin H synthase,⁴⁹

Chart II



and in the copper enzyme galactose oxidase.⁵⁰ A third possibility is that the diiron(III) peroxide unit itself might be sufficiently activated to carry out the hydroxylation reaction. As depicted in Scheme III, the peroxide ligand might perhaps be coordinated to the diiron(III) unit in an η^2, η^2 fashion, analogous to the binding mode thought to be important in dicopper enzymes that transport or activate dioxygen, for example, hemocyanin and tyrosinase.^{51,52} Other modes of peroxide binding to one or both iron atoms are possible, as indicated in Chart II. Several important model studies have provided spectroscopic evidence for the existence of diiron(III) peroxides;^{53,54} related species,⁵⁵⁻⁵⁷ including a high-valent iron oxo intermediate,⁵⁸ have been implicated in hydroxylation and epoxidation reactions. None has yet been structurally characterized, however.

Once the activated iron center, whatever it may be, is discharged, product is released with concomitant formation of the diiron(III) form of the hydroxylase that enters another cycle in the catalysis (Scheme II). A peroxide shunt, analogous to that found in the P-450 enzyme, has been reported for the *M. trichosporium* OB3b MMO¹⁶ and confirmed by us in the present work. As indicated in Scheme II, the use of hydrogen peroxide permits hydroxylation reactions to occur in the absence of the coupling and reductase proteins. Addition of hydrogen peroxide to hydroxylase isolated from the *M. capsulatus* (Bath) organism, however, displays only 5–10% of the activity measured with the complete, three-protein system.⁵⁹

Mechanistic Considerations: The Hydroxylation Reaction. Scheme IV presents six possible mechanisms for substrate hydroxylation following generation of the dioxygen-activated diiron center. In all cases except the last, O–O bond cleavage occurs prior to the C–H bond breaking step. The six possibilities are (A) direct insertion of the oxygen atom of a high-valent iron oxo species into a C–H bond; (B) concerted addition of the C–H bond to the high-valent iron oxo species to form a metal–carbon bond followed by reductive elimination of the alcohol; (C) heterolytic attack of a high-valent iron oxo species on the R–H bond followed by recombination to afford product; (D) homolytic attack of a high-valent iron oxo species on the R–H bond followed by return

(48) Bollinger, J. M., Jr.; Edmondson, D. E.; Huynh, B. H.; Filley, J.; Norton, J. R.; Stubbe, J. *Science* **1991**, *253*, 292–298.

(49) Kulmacz, R. J.; Ren, Y.; Tsai, A.-L.; Palmer, G. *Biochemistry* **1990**, *29*, 8760–8771.

(50) Babcock, G. T.; El-Deeb, M. K.; Sandusky, P. O.; Whittaker, M. M.; Whittaker, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 3727–3734.

(51) Nasir, M. S.; Cohen, B. I.; Karlin, K. D. *J. Am. Chem. Soc.* **1992**, *114*, 2482–2494.

(52) Kitajima, N.; Fujisawa, K.; Fujimoto, C.; Moro-oka, Y.; Hashimoto, S.; Kitagawa, T.; Toriumi, K.; Tatsumi, K.; Nakamura, A. *J. Am. Chem. Soc.* **1992**, *114*, 1277–1291.

(53) Kitajima, N.; Fukui, H.; Moro-oka, Y. *J. Am. Chem. Soc.* **1990**, *112*, 6402–6403.

(54) Ménage, S.; Brennan, B. A.; Juarez-Garcia, C.; Münck, E.; Que, L., Jr. *J. Am. Chem. Soc.* **1990**, *112*, 6423–6425.

(55) Tung, H.-C.; Kang, C.; Sawyer, D. T. *J. Am. Chem. Soc.* **1992**, *114*, 3445–3455.

(56) Barton, D. H. R.; Bèvière, S. D.; Chavasiri, W.; Cshai, E.; Doller, D. *Tetrahedron* **1992**, *48*, 2895–2910.

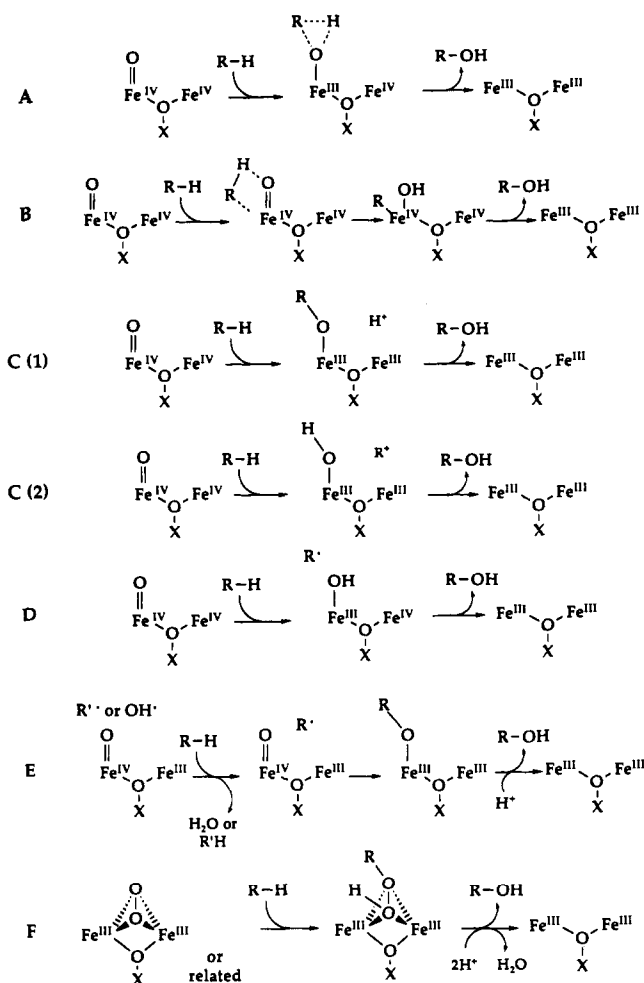
(57) Murch, B. P.; Bradley, F. C.; Que, L., Jr. *J. Am. Chem. Soc.* **1986**, *108*, 5027–5028.

(58) Leising, R. A.; Brennan, B. A.; Que, L., Jr. *J. Am. Chem. Soc.* **1991**, *113*, 3988–3990.

(59) Liu, K. E.; Lippard, S. J. Unpublished results.

(47) Fox, B. G.; Froland, W. A.; Dege, J. E.; Lipscomb, J. D. *J. Biol. Chem.* **1989**, *264*, 10023–10033.

Scheme IV



of the hydroxyl group from iron to the alkyl radical, a so-called oxygen rebound step; (E) abstraction of a hydrogen atom from the substrate by hydroxyl or another radical within the active site to form an alkyl radical, which then adds to the iron-bound oxygen atom; and (F) electrophilic attack of an η^2, η^2 -peroxide or related species (Chart II) on the substrate to form a carbon-oxygen bond followed by release of product. Mechanisms D and E involve transient formation of a substrate-derived alkyl radical, which could rearrange as shown in Scheme I, and it is these pathways that are directly addressed by the present investigation. The carbocation shown in mechanism C(2) could also rearrange, and its involvement in the hydroxylation reaction would similarly be revealed by our studies.

Experiments reported here and previously^{9,15} with MMO from *M. trichosporium* OB3b indicate that a radical is formed in the catalytic cycle, and a paper has recently appeared reporting results that are consistent with a radical or carbocationic intermediate.⁴⁵ No ring-opened products are observed during the hydroxylation of substrate probes 1–4 with MMO from *M. capsulatus* (Bath), however. There are several possible explanations for these results. The simplest is that radicals and carbocations do not form and that pathways C(2), D, and E can be excluded for the reaction mechanism for MMO from this organism. Alternatively, radicals could form but recombine rapidly with the high-valent iron oxo moiety before they have a chance to ring open. Although this alternative is reasonable for probes 3 and, possibly, 4, it is not a viable explanation for 1 and 2 since it would require a radical rebound rate constant greater than $4 \times 10^{13} \text{ s}^{-1}$. This conclusion is only valid, however, provided that the hydroxylase active site does not perturb the ring-opening rate constant of probes 1 and 2. Moreover, there are other potential problems arising in probe substrate oxidations within enzyme active sites, as delineated in a recent report of cytochrome P-450 dependent enzyme oxida-

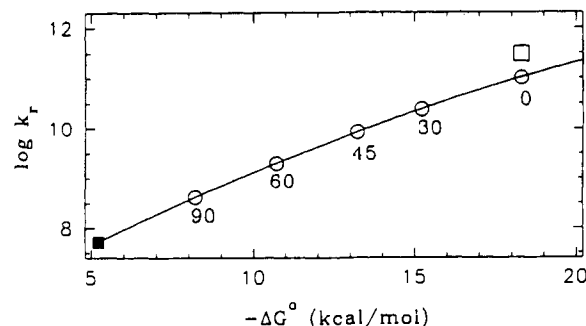


Figure 2. A plot of $\log k_r$ at 25 °C as a function of the exergonicity of the ring-opening reaction. The solid square is the experimental rate constant for cyclopropylcarbinyl radical ring opening, and the hollow square is that for the radical derived from 1. Predicted rate constants for ring opening of the latter as a function of the dihedral angle between the cyclopropyl and phenyl π -system planes are shown as open circles.

tions.²² In that study, two “poorly behaved” probes gave much less than the expected amount of rearranged product. In addition to decreasing the rate constant for radical rearrangement owing to steric effects, the low yields of rearranged products might arise from a possible change in mechanism, for example, the onset of an insertion reaction, an increase in the rate constant of oxygen rebound, or reaction of the rearranged radical with another site on the enzyme.

Two of these potential problems can be excluded as the reason for the failure to detect ring-opened alcohols in the present study. Because the rearrangement of radical U^\bullet to R^\bullet (Scheme I) is fast enough to compete with any other process, it is not possible that acceleration of the oxygen rebound step without a diminution in the rate constant for the radical rearrangement could subvert the rearrangement. Furthermore, if ring-opened radicals were to react with another position in the enzyme active site, then the probe would most likely have deactivated the enzyme as a suicide substrate inhibitor. Although an extensive analysis of enzyme activity was not conducted for these substrates, there did not appear to be a noticeable loss of activity over the 15-min time course in these studies.

The other possibility, that constraints of the enzyme active site alter the rate constant for radical rearrangement, is subtle and cannot be excluded. It is possible, for example, that the orientation of the phenyl group in enzyme-bound probe 1 is controlled by the steric confinement of the active site so as to preclude coalignment of the π -system of the phenyl ring with the bond of the cyclopropane ring that is cleaved. The imposition of such a stereoelectronic effect on the ring-opening reaction might result in a marked reduction in the rate constant for ring opening of the radical derived from 1. In the extreme case where the π -system of the phenyl ring is oriented perpendicularly to the cyclopropyl ring bond that is opened, the contribution of a phenyl resonance effect to the cyclopropyl-carbinyl bond breaking step would be eliminated, leaving only an inductive effect.

The manifestation of an active site constraint enforced stereoelectronic effect on the kinetics of ring opening can be estimated semiquantitatively via Marcus theory.^{60–62} We have shown⁴² that the rate constants for reactions of the parent system, ring opening of the cyclopropylcarbinyl radical (5) to the 3-butenyl radical (6) and cyclization of radical 6, can be used to predict rate constants for ring openings of substituted cyclopropylcarbinyl radicals. In brief, the rate constant for ring opening of 5 at 25 °C ($k_r = 5 \times 10^7 \text{ s}^{-1}$ for cleavage of one bond)^{64–67} and that for cyclization

(60) Lowry, T. H.; Richardson, K. S. *Mechanism and Theory in Organic Chemistry*, 3rd ed.; Harper & Row: New York, 1987, pp 222–227.

(61) Marcus, R. A. *Ann. Rev. Phys. Chem.* **1964**, *15*, 155–196.

(62) Applications of Marcus theory to predict the rate constants of group-transfer reactions of radicals are generally quite accurate when charge-transfer states are unimportant in the radical reactions.⁶³ A Marcus approach was used successfully to estimate rate constants for hydrogen atom transfer reactions.⁴⁰

(63) Fox, G. L.; Schlegel, H. B. *J. Phys. Chem.* **1992**, *96*, 298–302.

of 6 ($k_r = 8000 \text{ s}^{-1}$)⁶⁸ give $\Delta G^\circ = -5.2 \text{ kcal/mol}$; ΔG^\ddagger has been measured to be 6.9 kcal/mol. Solving the Marcus equation (eq 5) with these values gives $\Delta G^\ddagger_i = 9.32 \text{ kcal/mol}$, the intrinsic

$$\Delta G^\ddagger = \Delta G^\ddagger_i + \Delta G^\circ/2 + (\Delta G^\circ)^2/16\Delta G^\ddagger_i \quad (5)$$

activation energy expected for a thermoneutral cyclopropylcarbiny radical ring opening. This value of ΔG^\ddagger_i can be used in eq 5 to calculate ΔG^\ddagger for cyclopropylcarbiny ring openings of varying exergonicity. Figure 2 shows a plot of $\log k_r$ as a function of the exergonicity of the reaction.

Comparisons of standard bond dissociation energies (BDEs) can be used to estimate the exergonicity of the cyclopropylcarbiny ring-opening reactions. The BDE for a benzylic hydrogen in toluene is 10–11 kcal/mol smaller than that of a primary C–H bond in ethane or propane.^{71,72} For the benzylic position in ethylbenzene, the BDE is reduced by approximately 2.5 kcal/mol from that of toluene.^{71,72} Thus, the ring opening of the radical derived from 1 is expected to be about 12.5–13.5 kcal/mol more exergonic than that of 5, giving a value of $\Delta G^\circ = -18.2 \text{ kcal/mol}$. If we assume that the full overlap of the π -system of the phenyl ring is available in the transition state for ring opening of the radical derived from 1, then the predicted rate constant for ring opening of this radical at 25 °C is $1 \times 10^{11} \text{ s}^{-1}$. Given the crude nature of the analysis, this value compares favorably with the observed rate constant of $3 \times 10^{11} \text{ s}^{-1}$.

If the phenyl group in the radical derived from 1 is locked such that its π -system cannot overlap fully with the breaking bond in the cyclopropyl ring, the resonance stabilization would be a function of $\cos^2 \theta$, where θ is the dihedral angle of the breaking bond and the π -system of the phenyl ring. When the latter is orthogonal to the breaking bond ($\theta = 90^\circ$), the effect of the phenyl group would be reduced to approximately that of the inductive effect of an alkyl group. In this extreme case, the appropriate model reaction for the Marcus analysis would be one in which the exergonicity of the ring-opening reaction is only about 2.5–3 kcal/mol greater than that of 5 on the basis of the differences in BDE between primary and secondary alkane C–H bonds.⁷¹ This value gives a predicted rate constant at 25 °C of about $4 \times 10^8 \text{ s}^{-1}$.

Using the two limiting cases discussed above, corresponding to phenyl stabilization of between 3 and 13 kcal/mol, the stereoelectronic effect of an enforced position of the phenyl group in the radical derived from 1 is shown on the plot in Figure 2 for selected dihedral angles. When θ is 60° , the expected rate constant for ring opening at 25 °C is reduced to $2 \times 10^9 \text{ s}^{-1}$, which is about 1 order of magnitude less than the oxygen rebound rate constants found in P-450 oxidations.²² Because it is possible that an oxygen rebound rate constant in the MMO hydroxylase is faster than that found in the P-450 studies, further reduction of the rate constant for ring opening of the radical derived from 1 could lead to undetectably small amounts of ring-opened alcohol product 1b. Thus the absence of 1b from oxidation of probe 1 could result from an extreme alteration in the rate constant for ring opening of the derived cyclopropylcarbiny radical in the enzyme active site.^{73,74}

Although the Marcus analysis demonstrates that a stereoelectronic reduction in the rate constant for ring opening of a radical derived from 1 is a possible explanation for the absence of ring-opened alcohol 1b, it should be recalled that no ring-opened product formed in the hydroxylation of the closely related substrate 2. A significant reduction in the rate constant for ring opening of the radical derived from this substrate probe due to steric effects in the active site is less likely. In this case, both phenyl groups must be held such that their π -systems are virtually orthogonal to the breaking bond in order to reduce resonance stabilization in the transition state for opening, a much less probable situation. Moreover, such an arrangement would require that the phenyl groups approach coplanarity with one another, a geometry that would increase steric interactions between them. The results from probe 2 therefore suggest that a radical or carbocation is not formed and that the hydroxylation mechanism involves one of the alternative pathways A, B, C(1), or F in Scheme IV.

There is another factor to consider with regard to the results with probe 1. The discussion of steric effects on the rate constant for ring opening of substrate-derived radical ignored the fact that two enantiomers of this substrate were allowed to react with the chiral enzyme. If sterics-enforced stereoelectronic effects were operative, it is unlikely that both enantiomeric radicals derived from the enantiomers of the probe would suffer similar ring-opening rate retardations. The formation of aryl hydroxylated product in amounts comparable to the amount of alcohol 1a produced complicates the analysis, however. As indicated above, it is likely that the two different products are derived from the two different enantiomers of the probe substrate. Further studies with enantiomerically pure probes are planned to resolve this question.

Aside from steric factors within the active site, other considerations to explain the lack of rearranged substrate with *M. capsulatus* (Bath) must be addressed. For example, one cannot rule out that the hydroxylation reaction may proceed through two different mechanisms from a common intermediate. One pathway, D or E, could involve a substrate radical, thus accounting for the rearranged products observed with MMO from *M. trichosporium* OB3b. An alternative pathway could involve mechanism A, B, C, or F. The specific pathway chosen would then depend on the energetics of the substrate. It is possible, however, that the frequency with which each pathway is followed may differ between the two organisms. Alternatively, MMO from *M. capsulatus* (Bath) and *M. trichosporium* OB3b may exhibit different hydroxylation mechanisms, which would explain the presence of ring-opened products for the latter.

We now return to the intramolecular deuterium isotope effect of 5.1 observed for the hydroxylation of deuterated 1. Although an isotope effect of this magnitude is generally associated with processes that involve a relatively linear transition state,⁷⁵ such as would occur for oxygen rebound, mechanisms containing intramolecular cyclic transition states cannot be excluded. In fact, dimethyldioxirane, a reagent believed to oxidize hydrocarbons via an insertion process,⁷⁶ oxidized cyclododecane-*d*₀ and -*d*₂₄ with an isotope effect of 5.⁷⁷ Thus our observed value of 5.1 would be consistent with pathways A, B, C(1), and F, all of which involve transition states for C–H bond cleavage that resemble the dioxirane oxygen insertion process and avoid the formation of a substrate radical or carbocation in the catalytic cycle.⁷⁵ The

(64) Maillard, B.; Forrest, D.; Ingold, K. U. *J. Am. Chem. Soc.* **1976**, *98*, 7024–7026.

(65) Mathew, L.; Warkentin, J. *J. Am. Chem. Soc.* **1986**, *108*, 7981–7984.

(66) Newcomb, M.; Glenn, A. G. *J. Am. Chem. Soc.* **1989**, *111*, 275–277.

(67) Beckwith, A. L. J.; Bowry, V. W.; Moad, G. J. *Org. Chem.* **1988**, *53*, 1632–1641.

(68) The reported⁶⁹ rate constant for cyclization of the 3-butenyl radical has been corrected using a more recently determined value⁷⁰ for the rate constant for reaction of Bu₃SnH with a primary radical.

(69) Effio, A.; Griller, D.; Ingold, K. U.; Beckwith, A. L. J.; Serelis, A. K. *J. Am. Chem. Soc.* **1980**, *102*, 1734–1736.

(70) Johnston, L. J.; Luszyk, J.; Wayner, D. D. M.; Abeywickreyma, A. N.; Beckwith, A. L. J.; Scaiano, J. C.; Ingold, K. U. *J. Am. Chem. Soc.* **1985**, *107*, 4594–4596.

(71) McMillen, D. F.; Golden, D. M. *Ann. Rev. Phys. Chem.* **1982**, *33*, 493–532.

(72) Bordwell, F. G.; Cheng, J.-P.; Harrelson, J. A., Jr. *J. Am. Chem. Soc.* **1988**, *110*, 1229–1231.

(73) For a related treatment of stereoelectronic effects on the kinetics of radical reactions, see: Tanko, J. M.; Kamrudin, N.; Blackert, J. F. *J. Org. Chem.* **1991**, *56*, 6395–6399.

(74) Another possible explanation for formation of cyclic alcohol 1a, reversible ring opening of the radical from 1 and selective trapping of the cyclic radical, can be excluded readily. From the expected exergonicity of the ring-opening reaction (i.e., $\Delta G^\circ = -18.2 \text{ kcal/mol}$), the equilibrium constant for the ring opening at room temperature is about 2×10^{13} . With a ring-opening rate constant of $3 \times 10^{11} \text{ s}^{-1}$, the ring closure of the acyclic radical must have a rate constant on the order of 0.01 s^{-1} . That the benzylic radical could have a lifetime of 100 s at room temperature is without precedence, and the turnover rate of MMO exceeds this value.

(75) O'Ferrall, R. A. *M. J. Chem. Soc. B* **1970**, 785–790.

(76) Adam, W.; Curci, R.; Edwards, J. O. *Acc. Chem. Res.* **1989**, *22*, 205–211.

(77) Murray, R. W.; Jeyaraman, R.; Mohan, L. *J. Am. Chem. Soc.* **1986**, *108*, 2470–2472.

previous observation of an NIH shift in the hydroxylation of aromatic substrates with MMO from *M. capsulatus* (Bath) would support such a mechanism.^{8,78}

In summary, the failure of substrate probes **1** and **2** to form rearranged alcohol products upon hydroxylation with the *M. capsulatus* (Bath) MMO is best interpreted in terms of a mechanism that does not involve the formation of a radical or carbocation, namely, A, B, C(1), or F. It is unlikely that the enzyme active site induces a stereoelectronic barrier to ring opening. The fact that the same amount (85%) of the probe, both converted and unconverted, can be recovered from the enzymatic reaction mixture under hydroxylation conditions or in control reactions omitting NADH indicates that ring-opened substrate radical has not formed and been lost in the extraction procedure. In addition, these probes do not lead to inactivation of the enzyme over the time course of the reactions, indicating that ring-opened product has not formed and reacted with the enzyme. The kinetic isotope results are consistent with a concerted pathway for C-H bond insertion for the key hydroxylation step.

Comparisons with Related Work. The MMO mechanism is almost always drawn in analogy to that of cytochrome P-450.^{8,15,16} In particular, a catalytic cycle involving an iron oxo intermediate (Scheme III) that abstracts H[•] from the alkane (mechanism D, Scheme IV) has been proposed by several authors. The ability of high concentrations of hydrogen peroxide to effect a shunt pathway for the *M. trichosporium* OB3b MMO has been cited as evidence for heterolytic cleavage of the O-O bond,¹⁶ although it is noteworthy that, unlike the situation for cytochrome P-450, other oxo-transfer reagents could not be substituted for H₂O₂. Hydrogen peroxide does not operate at comparable levels in the *M. capsulatus* (Bath) system.⁵⁹ Various authors have invoked radical intermediates for the hydroxylation reaction. For example, *exo,exo,exo,exo*-2,3,5,6-*d*₄-norbornane epimerizes upon hydroxylation by *M. trichosporium* OB3b MMO,¹⁵ a result that has its parallels in cytochrome P-450 chemistry.⁷⁹ The extent of epimerization with MMO, however, was significantly less, being 2% following hydrogen atom abstraction from the endo position compared to 18% with cytochrome P-450, and 5% after abstraction at the exo position compared to 14% with cytochrome P-450. Similarly, allylic rearrangements occurred in 20% of the MMO hydroxylation products from 3,3,6,6-*d*₄-cyclohexene compared to 33% with cytochrome P-450.^{15,80} Mechanistic studies of MMO from *M. trichosporium* OB3b with the free radical probe 1,1-dimethylcyclopropane and with (*S*)- and (*R*)-[1-²H,1-³H]ethane were also consistent with radical formation, but cationic intermediates could not be ruled out.^{14,45} It has also been claimed that radical intermediates can be trapped during methane hydroxylation by the MMO of *M. capsulatus* (Bath).²⁴ In addition, the mechanism for olefin epoxidation by MMO from *M. trichosporium* OB3b differs from that believed to occur with cytochrome P-450.¹⁵ With cytochrome P-450, the 1-trans proton of propylene exchanges with solvent protons during turnover,⁸¹ leading to the hypothesis that the epoxidation mechanism involves oxametallocycles and iron carbene intermediates. In the reaction of propylene with MMO, however, no such exchange occurred.¹⁵

In view of these and the present results, it would appear that no single unifying mechanism can account for all of the data. Instead, it seems likely to us that more than one mechanism might

be operative, depending upon factors such as the steric demands of the substrate at the active site of the hydroxylase, the organism from which the MMO is isolated, and even the temperature at which the hydroxylation is carried out. There are differences in the coupling proteins obtained from the *M. capsulatus* (Bath) and *M. trichosporium* OB3b organisms,^{82,83} which could alter the properties of the system. Moreover, it should be recalled that the *M. capsulatus* (Bath) methanotroph was isolated from thermal waters and has its optimal monooxygenase activity at 45 °C, whereas the MMO from *M. trichosporium* OB3b is typically assayed at ambient temperature (23 °C). Further studies, involving additional mechanistic probes and the application of other methodologies, are currently in progress to provide further insight into these issues.

Conclusions. The major findings of this investigation may be summarized as follows:

1. The absence of ring-opened products in the hydroxylation of probes **1** and **2** suggests that the mechanism for MMO from *M. capsulatus* (Bath) may not involve radicals or carbocations, at least for these substrates.

2. Isotope-effect studies with deuterated probe **1** indicate that the hydroxylation step is not rate-determining in the overall MMO mechanism but that substantial C-H bond activation is involved in this step.

3. The observation that equimolar quantities of phenol and alcohol are produced in the hydroxylations of racemic mixtures of **1** and its trideuterated analogue implies that chiral discrimination in the enzyme active site can dictate the regioselectivity of the reaction.

4. A semiquantitative Marcus theory analysis was used to evaluate how the ring-opening rate constant of phenyl-substituted cyclopropanes can be tuned by changing the dihedral angle between the phenyl and cyclopropyl rings.

5. Several detailed proposals have been set forth for two discrete arcs of the MMO catalytic cycle (Scheme II), the activation of the reduced, diiron(II) form by dioxygen (Scheme III), and the subsequent hydroxylation step (Scheme IV). These considerations should serve as a valuable reference point for the interpretation of mechanistic results from diiron oxo and related monooxygenases as well as their model compounds.

6. Analysis of the present and literature results for the hydroxylation of hydrocarbons by MMO suggests that multiple mechanisms may be operative.

Acknowledgment. We are grateful to the National Institute of General Medical Sciences (Grant GM 32134 to S.J.L.) and to the National Science Foundation (Grant CHE-9117929 to M.N.) for support of this research. K.E.L. acknowledges NIH Training Grant CA09112 from the National Cancer Institute for support and MIT for a Clare Boothe Luce Scholarship. We also thank J. Stubbe, E. N. Jacobsen, and K. L. Taft for valuable discussions and T. Salifoglou for experimental assistance.

Supplementary Material Available: Full details of the synthesis and characterization of substrates and products, plots of the time dependence of product formation with substrates **1** and **3** (Figure S1), substrate **2** (Figure S2), and substrate **4** (Figure S3), mass spectral data for **4b**, and GC conditions for analysis (Table S1) (9 pages). Ordering information is given on any current masthead page.

(78) Tsuda, M.; Oikawa, S.; Okamura, Y.; Kimura, K.; Urabe, T.; Nakajima, M. *Chem. Pharm. Bull.* **1986**, *34*, 4457-4466.

(79) Groves, J. T.; McClusky, G. A. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 154-160.

(80) Groves, J. T.; Subramanian, D. V. *J. Am. Chem. Soc.* **1984**, *106*, 2177-2181.

(81) Groves, J. T.; Avaria-Neisser, G. E.; Fish, K. M.; Imachi, M.; Kuczkowski, R. L. *J. Am. Chem. Soc.* **1986**, *108*, 3837-3838.

(82) Rosenzweig, A. C.; Feng, X.; Lippard, S. J. In *IUCCP Symposium on Applications of Enzyme Biotechnology*; Plenum Press: College Station, TX, 1991; pp 69-85.

(83) Tsien, H.-C.; Hanson, R. S. *Appl. Environ. Microbiol.* **1992**, *58*, 953-960.