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Intermediate Q from Soluble Methane Monooxygenase Hydroxylates the Mechanistic Substrate Probe Norcarane: Evidence for a Stepwise Reaction

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Abstract: Norcarane is a valuable mechanistic probe for enzyme-catalyzed hydrocarbon oxidation reactions because different products or product distributions result from concerted, radical, and cation based reactions. Soluble methane monooxygenase (sMMO) from *Methylosinus trichosporium* OB3b catalyzes the oxidation of norcarane to afford 3-hydroxymethylcyclohexene and 3-cycloheptenol, compounds characteristic of radical and cationic intermediates, respectively, in addition to 2- and 3-norcaranols. Past single turnover transient kinetic studies have identified several optically distinct intermediates from the catalytic cycle of the hydroxylase component of sMMO. Thus, the reaction between norcarane and key reaction intermediates can be directly monitored. The presence of norcarane increases the rate of decay of only one intermediate, the high-valent bis-μ-oxo $Fe(IV)_2$ cluster-containing species compound **Q**, showing that it is responsible for the majority of the oxidation chemistry. The observation of products from both radical and cationic intermediates from norcarane oxidation catalyzed by sMMO is consistent with a mechanism in which an initial substrate radical intermediate is formed by hydrogen atom abstraction. This intermediate then undergoes either oxygen rebound, intramolecular rearrangement followed by oxygen rebound, or loss of a second electron to yield a cationic intermediate to which OH^- is transferred. The estimated lower limit of 20 ps for the lifetime of the putative radical intermediate is in accord with values determined from previous studies of sterically hindered sMMO probes.

The O_2-coupled hydroxylation of alkanes to alcohols is a challenging chemical reaction because of the chemical inertness of hydrocarbons, the kinetic barriers to molecular oxygen reactivity, and the increased reactivity of the products over the substrates. The most remarkable example of biologically mediated alkane hydroxylation is the O_2 coupled conversion of methane to methanol. This reaction is the first step in the metabolic pathway used by methanotrophic bacteria to oxidize methane as their only viable source of carbon and energy.[1] Two different enzymes, soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO), have been found to efficiently catalyze this reaction.[2,3] Due to its relative ease of purification, the majority of the studies designed to

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(1) Hanson, R. S.; Hanson, T. E. *Microbiol. Rev.* **1996**, *60*, 439−471.

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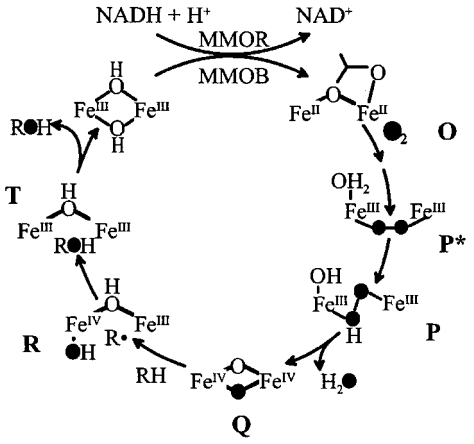
elucidate the mechanism of methane hydroxylation have focused on the sMMO,[4] in particular those isolated from the type II methanotroph *Methylosinus trichosporium* OB3b (sMMO OB3b) and the type X methanotroph *Methylococcus capsulatus* (Bath) (sMMO Bath).[5−7]

The sMMO is an iron-containing enzyme that is comprised of three different component proteins.[8,9] The hydroxylase component (MMOH) contains a bis-μ-hydroxo-bridged diiron[10−12] cluster that is essential for oxygen activation and substrate oxidation.[9] The reductase component (MMOR) transfers two electrons from NAD(P)H to the MMOH diiron cluster to prepare it for reaction with O_2.[13−16] The third component is a small effector protein termed component B (MMOB). MMOB has several roles in sMMO catalysis,[5−7] including an oxygen-gating effect that accelerates the rate of the reaction between the diferrous MMOH diiron cluster and molecular oxygen by nearly 1000-fold.[17] Recent studies have shown that MMOB also affects the rates of other steps throughout the catalytic cycle of MMOH.[18] Two electron reduced MMOH is capable of carrying out a single turnover of methane or any of the many adventitious substrates of sMMO to give the expected products in relatively high yield. However, efficient NAD(P)H coupled catalysis with 100% yield requires that all three components be present.[15,17]

Following the rapid reaction between diferrous MMOH and O_2 in the presence of MMOB, several reaction cycle intermediates have been shown to form and decay at progressively slower rates, allowing each to accumulate to levels sufficient to be observed and, in some cases, trapped.[5−7,19,20] Our current understanding of the sMMO catalytic cycle is shown in Scheme 1. Oxygen appears to react in two steps, forming first a putative Michaelis complex with the enzyme (compound **O**) and then a terminal superoxo or a bridging peroxo complex with the diiron cluster (compound **P***).[17,21,22] These intermediates have been detected in transient stopped-flow experiments and their optical

Scheme 1. The Catalytic Cycle of sMMO

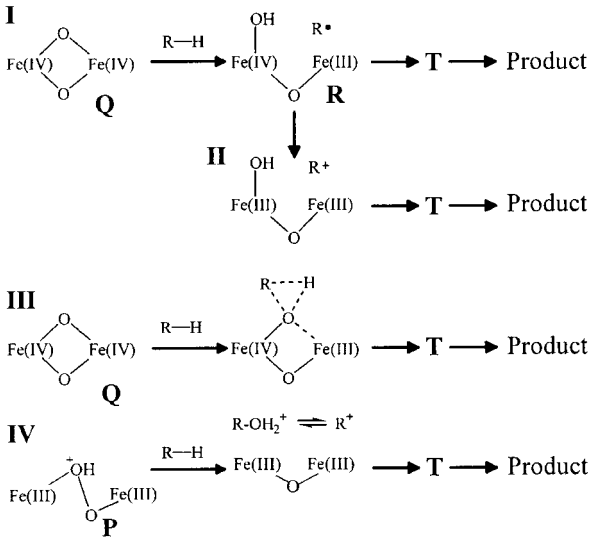


spectra have been shown to be similar to the weak spectrum of diferrous MMOH.[22] To date, these intermediates have not been trapped. In contrast, the next two intermediates, compound **P**[19,21,23] and compound **Q**,[20,24] have been trapped and characterized by using rapid freeze quench (RFQ) and transient kinetic techniques. Mössbauer and other spectroscopic studies of the enzyme and models have suggested that **P** contains a diferric μ-peroxo-bridged diiron cluster.[23,25] The UV/vis spectrum of **P** shows a broad absorbance maximum centered near 700 nm[21,26] as expected from the model complexes,[27] thus the formation and decay of **P** can be readily observed by using a stopped-flow spectrophotometer. Recently, diferric peroxo intermediates have been observed in other structurally related diiron enzymes.[28−30] Transient kinetic experiments designed to examine the sensitivity of the reaction time course to pH and solvent deuteration demonstrated that a proton is required in both the **P** formation and decay reactions, suggesting that **P** may actually be a hydroxoperoxo adduct, as shown in Scheme 1.[21]

Mössbauer[20,23,24] and EXAFS[24] spectroscopic studies have suggested that **Q** contains an unprecedented bis μ-oxo $Fe(IV)_2$ cluster in which the two single atom oxygen bridges form a so-called "diamond core". **Q** is electronically equivalent to the Fe(IV)═O π cation radical intermediate that is thought to be the reactive species in cytochrome P-450[31,32] and to compound I in heme-containing peroxidases.[33] **Q** has a bright yellow color (ϵ_{430} = 7.5 mM^{-1} cm^{-1}) and relatively slow formation and decay rate constants, facilitating detailed transient kinetic studies. The decay rate constant increases linearly with the concentration of substrate, and the apparent second-order rate constant from this process depends on the particular substrate employed,

(2) Anthony, C. *The Biochemistry of Methylotrophs*; Academic Press: London, 1982.
(3) Dalton, H. *Adv. Appl. Microbiol.* **1980**, *26*, 71−87.
(4) Abbreviations: sMMO, soluble form of methane monooxygenase; pMMO, particulate form of MMO; MMOH, hydroxylase component of sMMO; MMOR, reductase component of sMMO; MMOB, component B of sMMO; sMMO OB3b, sMMO isolated from *Methylosinus trichosporium* OB3b; sMMO Bath, sMMO isolated from *Methylococcus capsulatus* (Bath).
(5) Wallar, B. J.; Lipscomb, J. D. *Chem. Rev.* **1996**, *96*, 2625−2657.
(6) Brazeau, B. J.; Lipscomb, J. D. *Subcell. Biochem.* **2000**, *35*, 233−277.
(7) Merkx, M.; Kopp, D. A.; Sazinsky, M. H.; Blazyk, J. L.; Muller, J.; Lippard, S. J. *Angew. Chem., Int. Ed.* **2001**, *40*, 2782−2807.
(8) Colby, J.; Dalton, H. *Biochem. J.* **1978**, *171*, 461−468.
(9) Fox, B. G.; Froland, W. A.; Dege, J. E.; Lipscomb, J. D. *J. Biol. Chem.* **1989**, *264*, 10023−10033.
(10) Fox, B. G.; Surerus, K. K.; Münck, E.; Lipscomb, J. D. *J. Biol. Chem.* **1988**, *263*, 10553−10556.
(11) Elango, N.; Radhakrishnan, R.; Froland, W. A.; Wallar, B. J.; Earhart, C. A.; Lipscomb, J. D.; Ohlendorf, D. H. *Protein Sci.* **1997**, *6*, 556−568.
(12) Rosenzweig, A. C.; Frederick, C. A.; Lippard, S. J.; Nordlund, P. *Nature* **1993**, *366*, 537−543.
(13) Lund, J.; Dalton, H. *Eur. J. Biochem.* **1985**, *147*, 291−296.
(14) Lund, J.; Woodland, M. P.; Dalton, H. *Eur. J. Biochem.* **1985**, *147*, 297−305.
(15) Liu, Y.; Nesheim, J. C.; Paulsen, K. E.; Stankovich, M. T.; Lipscomb, J. D. *Biochemistry* **1997**, *36*, 5223−5233.
(16) Gassner, G. T.; Lippard, S. J. *Biochemistry* **1999**, *38*, 12768−12785.
(17) Liu, Y.; Nesheim, J. C.; Lee, S.-K.; Lipscomb, J. D. *J. Biol. Chem.* **1995**, *270*, 24662−24665.
(18) Wallar, B. J.; Lipscomb, J. D. *Biochemistry* **2001**, *40*, 2220−2233.
(19) Lee, S.-K.; Nesheim, J. C.; Lipscomb, J. D. *J. Biol. Chem.* **1993**, *268*, 21569−21577.
(20) Lee, S.-K.; Fox, B. G.; Froland, W. A.; Lipscomb, J. D.; Münck, E. *J. Am. Chem. Soc.* **1993**, *115*, 6450−6451.
(21) Lee, S.-K.; Lipscomb, J. D. *Biochemistry* **1999**, *38*, 4423−4432.
(22) Brazeau, B. J.; Lipscomb, J. D. *Biochemistry* **2000**, *39*, 13503−13515.
(23) Liu, K. E.; Valentine, A. M.; Wang, D. L.; Huynh, B. H.; Edmondson, D. E.; Salifoglou, A.; Lippard, S. J. *J. Am. Chem. Soc.* **1995**, *117*, 10174−10185.
(24) Shu, L.; Nesheim, J. C.; Kauffmann, K.; Münck, E.; Lipscomb, J. D.; Que, L., Jr. *Science* **1997**, *275*, 515−518.
(25) Kim, K.; Lippard, S. J. *J. Am. Chem. Soc.* **1996**, *118*, 4914−4915.
(26) Liu, K. E.; Wang, D.; Huynh, B. H.; Edmondson, D. E.; Salifoglou, A.; Lippard, S. J. *J. Am. Chem. Soc.* **1994**, *116*, 7465−7466.
(27) Du Bois, J.; Mizoguchi, T. J.; Lippard, S. J. *Coord. Chem. Rev.* **2000**, *200*−*202*, 443−485.
(28) Moenne-Loccoz, P.; Baldwin, J.; Ley, B. A.; Loehr, T. M.; Bollinger, J. M., Jr. *Biochemistry* **1998**, *37*, 14659−14663.
(29) Moenne-Loccoz, P.; Krebs, C.; Herlihy, K.; Edmondson, D. E.; Theil, E. C.; Huynh, B. H.; Loehr, T. *Biochemistry* **1999**, *38*, 5290−5295.
(30) Broadwater, J. A.; Ai, J.; Loehr, T. M.; Sanders-Loehr, J.; Fox, B. G. *Biochemistry* **1998**, *37*, 14664−14671.
(31) Groves, J. T.; Han, Y.-Z. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd ed.; Plenum: New York, 1995; pp 3−48.
(32) Groves, J. T. In *The Porphyrin Handbook*; Kadish, K. M., Smith, K. M., Guilard, R., Eds.; Academic Press: San Diego, CA, 2000; pp 17−40.
(33) Dawson, J. H. *Science* **1988**, *240*, 433−439.

Scheme 2. Four Possible Mechanisms of Substrate Hydroxylation by sMMO

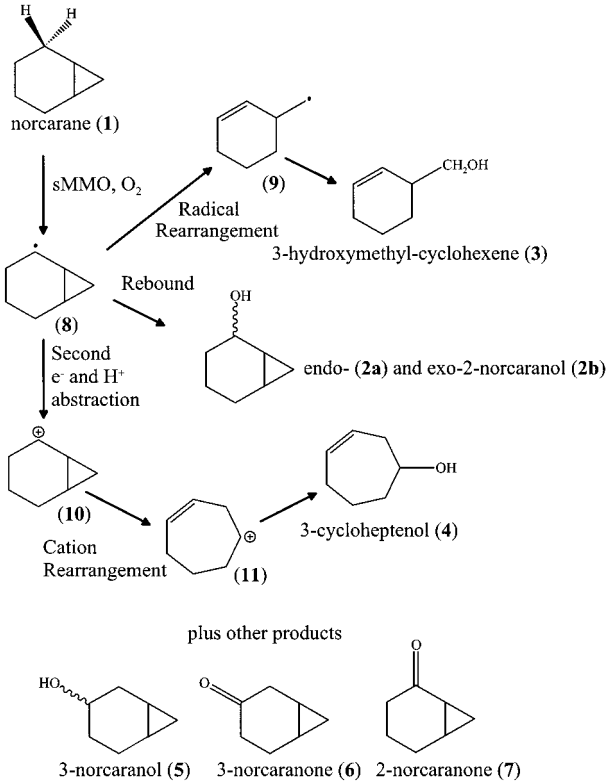


demonstrating that **Q** is the principle reactive species of the cycle.[19,21,22,34] As such, it represents the only example of an enzyme intermediate that can oxidize unactivated C−H bonds that has been trapped and characterized. The immediate product of the reaction of **Q** with substrate is an enzyme−product complex termed **T**, which subsequently releases the product in the rate-limiting step of the cycle.[19]

There is currently significant debate regarding the mechanism of the substrate oxidation by sMMO.[35−39] One reason that the nature of the mechanism remains clouded is that different interpretations of similar data from sMMO OB3b and sMMO Bath have been published. Most of the uncertainty derives from the interpretation of the results from studies involving various radical clock substrates.[40−44] Also, recent reports that **P** is capable of carrying out substrate oxidation reactions in some cases have raised the question of whether **Q** is the true reactive species, particularly for more easily oxidized substrates.[41,45]

Four fundamentally different reaction mechanisms for hydrocarbon hydroxylation by sMMO have been suggested (Scheme 2): (I) hydrogen atom abstraction from the substrate followed by radical recombination;[46] (II) cation formation by electron abstraction from the substrate radical intermediate generated in I followed by reaction with metal bound hydroxide;[47] (III) direct insertion of the oxygen atom from **Q** into the C−H bond;[41−43,48] and (IV) cation formation on the substrate by transfer of a protonated oxygen from a hydroperoxy intermediate (derived from O_2) followed by loss of water.[41] In principle, the possible hydroxylation mechanisms of Scheme 2 can be differentiated based upon the distinguishing feature of each: Mechanism I will have a substrate based radical intermediate and a large deuterium kinetic isotope effect, whereas these features will be absent for III. Mechanisms II and IV will both have a cationic substrate intermediate, but they can be differentiated based on the identity of the reactive species, **P** or **Q**, respectively. In practice, problems have been encountered in experimentally distinguishing between these four mechanisms. These problems may arise from a number of sources, including the following: (i) The use of diagnostic radical clock substrates that do not distinguish between radical and cation mechanisms. (ii) The use of probes with radical reorganization rate constants that are either too fast or too slow to "clock" the enzymatic process. (iii) The balance between enzyme and substrate specific steric and electronic interactions that determine much about the effective nature of the intermediate or transition state.

Scheme 3. The Oxidation of Norcarane (**1**) by sMMO and the Mechanism of Rearrangement of Transient Radical and Cation Intermediates[a]



[a] Products **2**−**7** were observed in the GC/MS chromatogram shown in Figure 1.

The use of the diagnostic probe norcarane (bicyclo[4.1.0]-heptane)[49] (**1**) presents an opportunity to address at least some of these issues because (i) distinct products result from radical and cation based intermediates (see Scheme 3), (ii) all of the substrate carbons should be similarly accessible to the active site reactive species, and (iii) distortion of **1** in the active site

(34) Nesheim, J. C.; Lipscomb, J. D. *Biochemistry* **1996**, *35*, 10240−10247.
(35) Lipscomb, J. D.; Que, L. *JBIC* **1998**, *3*, 331−336.
(36) Whittington, D. A.; Valentine, A. M.; Lippard, S. J. *JBIC* **1998**, *3*, 307−313.
(37) Deeth, R. J.; Dalton, H. *JBIC* **1998**, *3*, 302−306.
(38) Shteinman, A. A. *JBIC* **1998**, *3*, 325−330.
(39) Yoshizawa, K. *JBIC* **1998**, *3*, 318−324.
(40) Jin, Y.; Lipscomb, J. D. *Biochemistry* **1999**, *38*, 6178−6186.
(41) Choi, S.-Y.; Eaton, P. E.; Kopp, D. A.; Lippard, S. J.; Newcomb, M.; Shen, R. *J. Am. Chem. Soc.* **1999**, *121*, 12198−12199.
(42) Liu, K. E.; Johnson, C. C.; Newcomb, M.; Lippard, S. J. *J. Am. Chem. Soc.* **1993**, *115*, 939−947.
(43) Valentine, A. M.; LeTadic-Biadatti, M. H.; Toy, P. H.; Newcomb, M.; Lippard, S. J. *J. Biol. Chem.* **1999**, *274*, 10771−10776.
(44) Jin, Y.; Lipscomb, J. D. *Biochim. Biophys. Acta* **2000**, *1543*, 47−59.
(45) Valentine, A. M.; Stahl, S. S.; Lippard, S. J. *J. Am. Chem. Soc.* **1999**, *121*, 3876−3887.
(46) Fox, B. G.; Borneman, J. G.; Wackett, L. P.; Lipscomb, J. D. *Biochemistry* **1990**, *29*, 6419−6427.
(47) Jin, Y.; Lipscomb, J. D. *JBIC* **2001**, *6*, 717−725.
(48) Valentine, A. M.; Wilkinson, B.; Liu, K. E.; Komarpanicucci, S.; Priestley, N. D.; Williams, P. G.; Morimoto, H.; Floss, H. G.; Lippard, S. J. *J. Am. Chem. Soc.* **1997**, *119*, 1818−1827.
(49) Groves, J. T.; Kruper, W. J., Jr.; Haushalter, R. C. *J. Am. Chem. Soc.* **1980**, *102*, 6375−6377.

should be minimal.[50−52] The value of **1** as a diagnostic substrate was recently demonstrated in a study of the alkane-metabolizing non-heme diiron monooxygenase AlkB in whole cells,[53,54] which generated a norcarane radical intermediate with a lifetime of approximately 1 ns.

In this study, we have investigated the reaction between purified sMMO from *M. trichosporium* OB3b and **1**. The results show that **1** is a good substrate for sMMO and that it reacts specifically with **Q**, the first direct demonstration of a radical clock reacting with a high valent species. Significantly, hydroxylation of **1** gives small amounts of products characteristic of radical and cation intermediates in addition to the principle endo- and exo-norcaranol products. This is consistent with a multistep mechanism in which hydroxylation proceeds via substrate intermediates that derive from hydrogen atom abstraction or hydrogen atom abstraction plus electron-transfer processes as proposed in mechanisms I and II.

Materials and Methods

Chemicals. NADH was purchased from Sigma and was purified to remove any contaminating ethanol as described previously.[55] The syntheses of norcarane and the authentic standards were based on well-established procedures. The syntheses of these compounds and their MS fragmentation patterns are described elsewhere.[56]

Purification of MMOH and MMOB. MMOH and MMOR were purified from *M. trichosporium* OB3b as previously described.[9] Recombinant MMOB was purified from *E. coli* strain BL21/DE3 containing plasmid pBWJ400 as previously described.[57]

Multiple Turnover Reactions of Norcarane by sMMO. Substrate hydroxylation reactions were performed by using the purified components of sMMO (MMOH, MMOB, MMOR). In a typical experiment, 20 μL of **1** was stirred in 5 mL of 50 mM MOPS buffer (pH 7.5) in a glass, Teflon sealed, reaction vial for 3 h at 23 °C. A 50 μL aliquot of this norcarane-saturated buffer solution was added to 450 μL of buffer containing the three protein components, each present at 25 μM. After the substrate was incubated with the enzyme for 15 min, the reaction was initiated with 4 μL of 297 mM ethanol free NADH. The reaction was stirred for 35 min at 23 °C and then quenched with an equal volume of GC-grade chloroform or ethyl acetate. The resulting mixture was vortexed for 1 min and then centrifuged for 2 min. The organic layer was analyzed on a HP GC/MS instrument (column HP-5MS, cross-linked 5% PH ME Siloxane) after evaporating 90% of the solvent with a gentle stream of argon gas. Products were identified based upon a comparison of the observed peaks with the retention times and fragmentation patterns of authentic standards.

Control experiments were done by mixing all reaction components except either NADH or MMOH. No conversion of **1** was observed. Additional control experiments were done by using the potential products in place of **1** in the enzyme-catalyzed reaction described above. There were no conversions of these products.

Single Turnover Reactions of Norcarane by sMMO. MMOH was reduced anaerobically as described below. Anaerobic, reduced MMOH was rapidly mixed with a solution containing a stoichiometric amount of MMOB, 250 μL of norcarane saturated buffer, and O_2 saturated 50 mM MOPS (pH 7) to a final volume of 1.5 mL at 23 °C. The final concentration of MMOH and MMOB was 213 μM active sites and 213 μM, respectively. The reaction was quenched after 1 min and worked up as described above for the multiple turnover reaction.

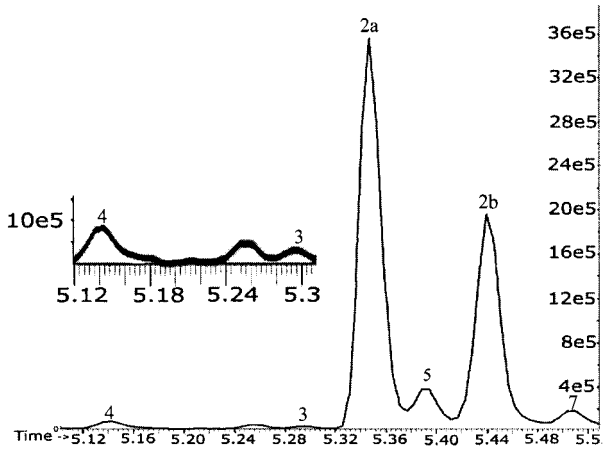


Figure 1. Gas chromatogram of the reaction mixture from sMMO and norcarane: **4** is 3-cycloheptenol; **3** is 3-hydroxymethylcyclohexene; **2a** is *endo*-2-norcaranol; **5** is *exo*-3-norcaranol; **2b** is *exo*-2-norcaranol (**5** may contain a small amount of unresolvable *endo*-3-norcaranol); **7** is 3-norcaranone.

Single Turnover Stopped-Flow Experiments. Single turnover experiments in the stopped-flow spectrophotometer (Applied Photophysics SX.MV18) and data analysis were performed as previously described.[19,21] In brief, 60 μM MMOH (active sites concentration) was made anaerobic by sparging with argon and reduced with a stoichiometric amount of dithionite with methyl viologen as the mediator dye. The reduced MMOH solution was transferred anaerobically to the stopped-flow apparatus and rapidly mixed with a solution containing 60 μM MMOB, oxygen, and **1**. The formation and decay of **Q** was observed at 430 nm. All experiments were performed at 12 °C in 50 mM MOPS, pH 7. The reciprocal relaxation times for the summed exponentials that describe the reaction time course were correlated with the rate constants for **Q** formation (**P** decay) and **Q** decay based upon an analysis described elsewhere.[19,21,34] Single turnover experiments following the formation and decay of **P** were followed at 700 nm and done in the same manner as those described above for **Q**. The three-exponential fit to the **P** time course was done as previously described.[21,22]

Results

MMO Catalyzed Oxidation of Norcarane. Turnover of **1** was demonstrated by incubation with the reconstituted sMMO system, NADH, and O_2 as described in the Material and Methods section. After extraction and separation by GC/MS, the well-resolved chromatogram of norcarane oxidation products shown in Figure 1 was obtained. The reaction products were identified by a comparison of their retention times and fragmentation patterns to those of authentic standards and controls. On average, approximately 57% of the total amount of norcarane-derived products was endo-2-norcaranol (**2a**), while 29% was exo-2-norcaranol (**2b**). Smaller amounts of 3-hydroxymethylcyclohexene (**3**), 3-cycloheptenol (**4**), exo-3-norcaranol (**5**), 3-norcaranone (**6**), and 2-norcaranone (**7**) were also detected as summarized in Table 1.

Identification of the Reactive MMOH Intermediate. To identify whether **P** or **Q** was the reactive species, single turnover experiments with **1** were done. The reaction was followed at 430 nm because this is the most sensitive wavelength at which to monitor both the **P** decay (**Q** formation) and **Q** decay reactions. It was expected that the rate constant for the decay of the species that reacts with **1** would be increased when this

(50) Friedrich, E. C.; Holmstead, R. L. *J. Org. Chem.* **1972**, *37*, 2546−2550.
(51) Rahm, A.; Amardeil, R.; Degueil-Castaing, M. *Organomet. Chem.* **1989**, *371*, C4−C8.
(52) Carlsson, D. J.; Ingold, K. U. *J. Am. Chem. Soc.* **1968**, *90*, 7047−7055.
(53) Austin, R. N.; Chang, H.-K.; Zylstra, G. J.; Groves, J. T. *J. Am. Chem. Soc.* **2000**, *122*, 11747−11748.
(54) R. N. Austin and J. T. Groves have recently found similar results for the non-heme diiron enzyme xylene monooxygenase: manuscript in preparation.
(55) Fox, B. G.; Froland, W. A.; Jollie, D. R.; Lipscomb, J. D. *Methods Enzymol.* **1990**, *188*, 191−202.
(56) R. N. Austin and J. T. Groves: manuscript in preparation.
(57) Chang, S. L.; Wallar, B. J.; Lipscomb, J. D.; Mayo, K. H. *Biochemistry* **1999**, *38*, 5799−5812.

Table 1. Product Distribution from sMMO Oxidation of Norcarane

product	% relative yield[a] (range)
endo-2-norcaranol (**2a**)	57 (53−64%)
exo-2-norcaranol (**2b**)	29 (24−32%)
3-hydroxymethyl-cyclohexene (**3**)	1.4 (0.4−3%)
3-cycloheptenol (**4**)	1.1 (0.6−1.7%)
3-norcaranol (**5**)	7 (6−7.7%)
3-norcaranone (**6**)[b]	2 (0.8−4%)
2-norcaranone (**7**)[b]	2.8 (1.5−4.9%)

[a] Represents the average of 3 runs. [b] Ketone products were not observed in single turnover reactions.

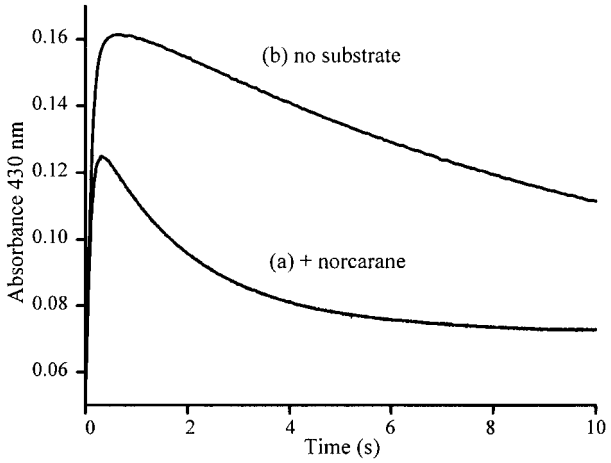


Figure 2. The effects of norcarane on the time course of **Q** formation and decay (a) with norcarane and (b) without norcarane at 430 nm, pH 7, 12 °C.

substrate is present. The time course of **Q** formation and decay is shown with and without **1** in Figure 2, curves a and b, respectively. The rate constant of **Q** formation in the absence of **1** ($10.7 \pm 0.5\ s^{-1}$) does not change in its presence ($10.3 \pm 0.1\ s^{-1}$) within the error or the measurement. In contrast, the rate constant of **Q** decay when reacted with **1** ($0.53 \pm 0.04\ s^{-1}$) is much faster than that of **Q** decay without **1** ($0.09 \pm 0.005\ s^{-1}$). In a separate experiment at 28 °C, the rate of **Q** decay doubled when the concentration of **1** was doubled, whereas the rate of **Q** formation was again unaffected (data not shown). As discussed above, previous studies have shown that **Q** is formed directly from **P**. The fact that the rate constant for **Q** formation does not depend on the norcarane concentration, while the rate constant for its decay is strongly dependent, indicates that the great majority of **1** is reacting directly with **Q**. This makes it unlikely that the hydroperoxo precursor, **P**, is a dominant reactant in the chemistry we are reporting.

In support of this conclusion, the single turnover reaction was also followed at 700 nm, the absorbance maximum for **P** from sMMO OB3b. By monitoring the reaction at this wavelength where the absorbance due to **Q** is greatly decreased, the formation and decay rate constants of **P** can be much more accurately determined. The time course at 700 nm is shown in Figure 3. It has previously been shown that the time course for **P** contains three-exponential phases; the reciprocal relaxation times correlate with the rate constants for **P** formation, **P** decay (**Q** formation), and **Q** decay, respectively.[21] By using the same rate constants for **Q** formation and decay as obtained from the 430 nm data ($10.3\ s^{-1}$ and $0.53\ s^{-1}$) plus a third rate constant of $34.2\ s^{-1}$, an excellent fit was obtained for the **P** time course. In the absence of **1**, the same formation and decay rate constants within error were found for **P**, but the smaller rate constant characteristic of **Q** autodecay was required to fit the time course.

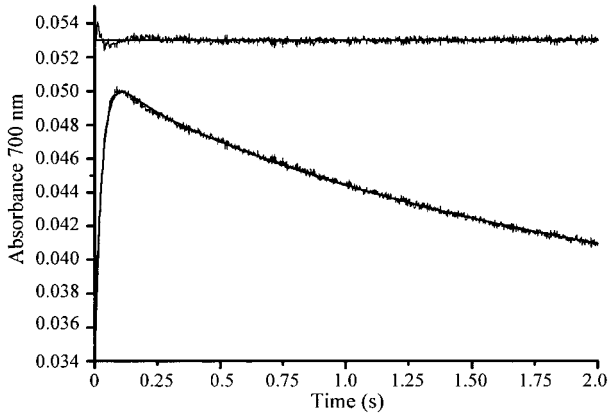


Figure 3. The time course of a single turnover reaction in the presence of norcarane observed at 700 nm, pH 7, 12 °C. The residual shown at the top is from a three-exponential fit utilizing the following rate constants for **P** formation, **P** decay, and **Q** decay: $34.2\ s^{-1}$, $10.3\ s^{-1}$, and $0.53\ s^{-1}$, respectively.

Products from a Single Turnover. Following reaction of 225 nmol of diferrous MMOH (active sites) and a stoichiometric amount of MMOB with O_2 and norcarane, the products from a single turnover could be detected. Although the quantities of products formed by this approach were too low for precise quantitation, GC/MS analysis clearly showed the formation of products **2a**, **2b**, **3**, **4**, and **5**. The ketone products **6** and **7** were not detected.

Discussion

Here we have studied single and multiple turnover reactions of sMMO with **1** as a diagnostic substrate in order to evaluate the oxidation mechanism and determine the reactive enzyme intermediate. Several products were observed and identified by comparison to authentic standards. The implications of these results for the mechanism of sMMO are discussed here.

Norcarane as a Diagnostic Probe. Norcarane can potentially distinguish between concerted, radical, and cationic pathways,[49−51] because each leads to a different product or product distribution as illustrated in Scheme 3. In a radical pathway, oxygen radical rebound would yield a mixture of **3** and **2a** + **2b** depending upon whether the ring-opening rearrangement has occurred or not. The 2-norcaranyl radical (**8**) rearranges to form the 3-cyclohexenylmethylradical (**9**) with a rate constant of approximately $2 \times 10^8\ s^{-1}$ at 25 °C.[53] In a cationic pathway, the 2-norcaranyl cation exists as resonance isomers between **10** and a seven-membered-ring species **11**. OH^- rebound (or transfer of H_2O followed by loss of H^+) would yield **2a**, **2b**, and **4**. In contrast to the stepwise radical and cation mechanisms, a concerted direct insertion reaction (e. g. **III)** would not yield rearranged products unless the required structural changes can occur during the lifetime of the transition state.[43] Direct insertion of a protonated oxygen from a hydroperoxide intermediate (e. g. **IV)** may also lead to the products from a cation intermediate, but no products from a radical intermediate would be observed.

Products from Stepwise Reactions are Observed. The presence of products **3** and **4,** which arise from radical and cationic intermediates, respectively, strongly suggests that the reaction proceeds at least in part by a stepwise mechanism (e.g. I, II, or IV**)**. Neither mechanisms I nor IV can be the exclusive pathway because products from both radical and cationic intermediates are observed and these mechanisms would each predict only one of the two types of intermediates. Mechanism III would not be expected to yield any radical or cation

intermediate, and thus it does not exclusively describe the oxidation reaction. Only a combination of mechanisms I and II can account for all of the products with a single reaction pathway (see further discussion below).

The major product alcohols from the reaction, **2a** and **2b**, are expected to arise from each mechanism I−IV discussed above, but are mechanistically diagnostic when found in conjunction with radical product **3**. Products in which **1** has been hydroxylated at the 3-position do not contribute to detecting or clocking intermediates; the low yield of these products is consistent with a mechanism that is sensitive to the C−H bond strength. The small amounts of ketones formed do not obscure our mechanistic interpretation because all ketones can be unequivocally identified by comparison to authentic standards and related back to the parent alcohol for purposes of quantification. No ketone products were observed in control reactions in which the alcohol products were used as substrates. Thus, one possible explanation for the presence of the ketone products is that they arise from second oxidation reactions of products in the active site before they are released from the enzyme. We have recently shown this to occur for another radical clock MMO substrate *cis*-1,2-dimethylcyclopropane, which is slowly released from the active site after initial oxidation.[44] In support of this hypothesis, we note that no ketone products were observed for norcarane oxidation in a single turnover reaction in which the second oxidation cycle in prohibited.

Control experiments allow us to report all identified products peaks with confidence. The possibility of abiotic oxidation either in the reaction mixture or in the GC injector port, the possibility of spurious Fenton-like chemistry from a resting state enzyme, the possibility that initial oxidation products are further transformed, and the possibility of abiotic isomerization during workup of the GC were all considered. Control experiments in which all reaction components except sMMOH or NADH were added to a reaction vessel showed no formation of any products (**2**−**7**) allowing us to rule out abiotic oxidation or Fenton-like chemistry. Control experiments in which potential products (**2**−**4**) were added in place of **1** showed no metabolism by sMMO allowing us to rule out abiotic and biotic isomerization. In addition, other experiments were performed[58] which showed that a ruthenium porphyrin oxidized **1** cleanly to **2** with no products of **3** or **4**, again confirming that the detection of **3** and **4** reported in these experiments is characteristic of the mechanism of norcarane oxidation by sMMO.

Compound Q is the Major Reactive Species. The single turnover data indicate that **Q** is the reactive species because it is the only intermediate for which a large increase in decay rate constant occurs when **1** is present. This means that mechanism IV, which relies on the reactivity of **P**, is unlikely to account for a significant fraction of the reaction products.

Evaluation of Mechanism 4 as a Minor Pathway. In the present study, we detect the product **4** from the cationic intermediate to be between 0.6 and 1.7% of the total reaction products. This value is within the error of the transient kinetic experiment that showed no change in the decay rate of **P**, thus a minor participation by mechanism IV cannot be ruled out as the source of this product. However, it is likely that the actual amount of cationic intermediate that is formed is much higher than 0.6−1.7%. In aqueous acetone at 80 °C, the distribution of products from the norcarane cationic intermediate that is formed is **2a** (68%), **2b** (21%), and **4** (11%).[59] This distribution will be sensitive to temperature, protonation state of the cation, and potentially steric and electronic constraints when the reaction occurs in the active site of an enzyme. Nevertheless, if the norcaranol:cycloheptenol ratio in the enzyme catalyzed reaction is even only half that observed in solution, the yield of the cationic intermediate would exceed the error in the transient kinetic experiment and allow mechanism IV to be eliminated from consideration.

Interpreting the Product Distribution. As discussed above, the presence of **3** and **4** is most consistent with stepwise processes such as those suggested by mechanisms I and II. Observed variability in the relative amount of radical product **3** produced (between 0.4 and 3%) coupled with our uncertainty about what percentage of **2a** and **2b** comes from a cationic precursor makes precise clocking of the radical lifetime difficult. The most conservative estimate, however, in which we use data from a reaction with a well-resolved chromatogram with the least relative amount of **3** (0.4%, Figure 1) and assume that all the 2-norcaranol produced comes from a radical intermediate yields an estimate for the minimum radical lifetime of 20 ps. At the other extreme, using a sample that generated a relatively large amount of radical product (3%) and assuming that for each molecule of **4** detected, 9 molecules of **2** are produced yields an estimate for the radical lifetime of 0.1 ns. All lifetimes in this range are significantly shorter than the nanosecond lifetime detected with AlkB, but still long enough to be considered a discrete radical intermediate rather than a component of a transition state. The hypothesis that **4** forms from rearrangement of a cation generated when the one electron reduced form of **Q** (an hydroxo-Fe(III)Fe(IV) enzyme species termed intermediate **R**) abstracts an electron from 2-norcaranyl radical requires only the very reasonable assumption that the second electron transfer occurs with a rate constant of approximately 1×10^{-10} s^{-1}.

Relevance to Methane Turnover. The use of a combination of mechanisms I and II to account for the reaction products from sMMO catalyzed norcarane turnover raises the question of whether methane is oxidized by the same route. The large KIE observed when sMMO catalyzes methane oxidation is compatible with the combination of mechanisms I and II in which complete C−H bond breaking occurs. Intermediate **R** is unlikely to be a strong enough oxidant to abstract an electron from a methane radical, therefore the observation of a cationic intermediate with norcarane may not reflect what occurs with methane hydroxylation. This is in accord with recent density functional calculations which predicted that **Q** can abstract a hydrogen atom from methane, but **R** can only abstract a hydrogen atom (or its equivalent) from ethane radical and compounds with weaker C−H bonds.[60]

Correlation with Other Studies of sMMO Mechanism. In past studies, the mechanism of sMMO has been probed with several different types of diagnostic substrates. These include chiral R and S 1-[^{2}H,^{3}H]-ethane,[48,61] a variety of cyclopropane derivatives,[41−44] methylcubane,[40,41] and substrates that undergo both desaturation and oxidation during sMMO turnover.[47] Within each class of probe molecules there is evidence for the formation of radical and or cationic intermediates. Chiral ethane undergoes 35% inversion during turnover.[48,61] 1,1,2,2-Tetramethylcyclopropane exhibits a small amount of rearrangement and ring opening prior to hydroxylation.[44] Methylcubane rearranges in a reaction characteristic of formation of a cation intermediate before hydroxylation.[40,41] Finally, 1,3-cyclohexa-

(58) Results of Mr. Chuanqing Wang, Princeton University, to be reported elsewhere.

(59) Friedrich, E. C.; Jassawalla, J. D. C. *J. Org. Chem.* **1979**, *44*, 4224−4229.

(60) Siegbahn, P. E. M. *JBIC* **2001**, *6*, 27−45.

(61) 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.

diene is converted to benzene (and hydroxylation and epoxidation products) during turnover consistent with a dehydrogenation that is an effective two electron oxidation, probably occurring in two successive one-electron-transfer steps.[47] In several cases, both radical and cation rearrangements have been observed, including the sMMO Bath catalyzed oxidation of *cis*-1-methoxy-2-phenyl-*trans*-methylcyclopropane[41] as well as sMMO OB3b catalyzed oxidation of 1,1-dimethylcyclopropane.[62] Similarly, ethylbenzene is converted to a mixture of products that includes 1- and 2-phenylethanols and styrene during turnover, consistent with the formation of radical and cationic intermediates.[47] Although these results are all consistent with mechanism I or II or their combination, the sMMO mechanism remains controversial because many of the cyclopropane radical clocks fail to show rearranged products. Also, the calculated radical lifetimes for some of the cyclopropane clocks that do exhibit rearrangement, as well as for chiral ethane inversion, are too short to be considered indicative of a discrete radical intermediate.[43] We have pointed out that all of the probes to date that fail to report the presence of discrete substrate intermediates are sterically unhindered at the position of oxidation, so they can potentially approach close to, and interact with, the activated diiron cluster.[44] Analogous results have been seen in model systems. With chiral binaphthyl iron porphyrin, stereoselective oxidation was seen on one face of the catalyst but not on the other, consistent with our hypothesis that steric interactions can affect radical clock products.[63] In contrast to the unhindered diagnostic substrates, probes that are more hindered at the position of oxidation all exhibit evidence for an intermediate with consistent lifetimes in the 30−500 ps range in the sMMO catalyzed reaction. Finally, the possibility of further oxidation of a radical intermediate[44,60] may contribute to both the failure to observe radicals in some cases and their short apparent lifetimes in others.

Norcarane has many favorable properties for use as a diagnostic substrate. All of its carbons are somewhat sterically hindered compared with a methyl group, increasing the probability of detecting an intermediate species if the hypothesis described above is correct. The availability of all of the possible products of norcarane oxidation as authentic standards allows them to be unambiguously identified and quantitated. Moreover, the fact that the rearrangement rate of a radical intermediate formed from **1** is 4−5 orders of magnitude longer than the lifetime of a transition state allows the detection of the product from radical rearrangement to firmly ascribe the reaction mechanism to one with a discrete intermediate. The lifetime of the radical intermediate we observe appears to be in the same range that we have reported for other sterically hindered substrates that could not be characterized in the same detail. The fact that sMMO produces much less of the products characteristic of radical or cationic intermediates in comparison with other enzyme catalyzed norcarane oxidations is consistent with the general observation from all of the probes utilized thus far with sMMO that the intermediate is quenched more rapidly than in other oxygenases.

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

(63) Groves, J. T.; Viski, P. *J. Am. Chem. Soc.* **1989**, *111*, 8537−8538.

Conclusion

Norcarane has proven to be a good substrate for sMMO. The products of norcarane metabolism include those characteristic of the rearrangements of both substrate based radical and cationic intermediates. Transient kinetic experiments indicate that **Q** is the dominant reactant in this chemistry and a mechanism in which the transfer of a second electron from the substrate competes with oxygen rebound has been proposed. The competing pathway of the transfer of the second electron presents a new complexity to analysis of the mechanism of sMMO, but also may account for the lack of the observation of radical or cation rearranged products in some previous studies.

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