

Oxidation of Ultrafast Radical Clock Substrate Probes by the Soluble Methane Monooxygenase from *Methylococcus capsulatus* (Bath)*

(Received for publication, November 18, 1998, and in revised form, January 19, 1999)

Ann M. Valentine‡, Marie-Helene LeTadic-Biadatti§, Patrick H. Toy§, Martin Newcomb§, and Stephen J. Lippard¶¶

From the ‡Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and §Department of Chemistry, Wayne State University, Detroit, Michigan 48202

Radical clock substrate probes were used to assess the viability of a discrete substrate radical species in the mechanism of hydrocarbon oxidation by the soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus* (Bath). New substituted cyclopropane probes were used with very fast ring-opening rate constants and other desirable attributes, such as the ability to discriminate between radical and cationic intermediates. Oxidation of these substrates by a reconstituted sMMO system resulted in no rearranged products, allowing an upper limit of 150 fs to be placed on the lifetime of a putative radical species. This limit strongly suggests that there is no such substrate radical intermediate. The two enantiomers of *trans*-1-methyl-2-phenylcyclopropane were prepared, and the regioselectivity of their oxidation to the corresponding cyclopropylmethanol and cyclopropylphenol products was determined. The results are consistent with selective orientation of the two enantiomeric substrates in the hydrophobic cavity at the active site of sMMO, specific models for which were examined by molecular modeling.

The soluble methane monooxygenase (sMMO)¹ from *Methylococcus capsulatus* (Bath), the hydroxylase component of which contains a dinuclear nonheme iron center, effects the mixed function oxidation of substrate,



Equation 1 (1–3). In methanotrophic bacteria from which sMMO is isolated, methane is the natural substrate, providing both carbon and energy for the growth of the cell. The kinetically difficult selective transformation of methane to methanol is carried out at the active site of a 251-kDa hydroxylase enzyme (MMOH) (4–7), the structure of which has been determined by x-ray crystallography. Electrons are supplied from NADH via a 38.5-kDa reductase protein (MMOR) (4, 8), and the reactivity of the system is modulated by a 15.8-kDa coupling protein (MMOB) (9, 10).²

* This work was supported in part by Grants GM32134 (to S. J. L.) and GM48722 (to M. N.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶¶ To whom correspondence should be addressed: Dept. of Chemistry, Massachusetts Institute of Technology, Rm. 18-590, Cambridge, MA 02139.

¹ The abbreviations used are: sMMO, soluble methane monooxygenase; MMOH, 251-kDa hydroxylase enzyme; MMOR, 38.5-kDa reductase protein; MMOB, 15.8-kDa coupling protein.

² G. T. Gassner, personal communication.

In addition to methane, sMMO can oxidize a wide variety of substrates (12–15). This property has allowed the use of radical clock substrate probes to derive mechanistic information about the hydroxylation step in the enzyme mechanism. Radical clocks were first described in 1980 (16) and calibrated (17) for use in mechanistic studies of proteins (18, 19) and related model compounds (20, 21). Specifically, these substrates can reveal whether radical intermediates are formed during a reaction and, if so, can establish their lifetime and the rate constant for radical rebound. Fig. 1 depicts the oxidation of a radical clock substrate probe, which uses the ring opening of a cyclopropylcarbinyl radical. If hydrogen atom abstraction from the probe occurs, the resulting radical can either rebound to form the unrearranged alcohol or undergo ring opening before rebound to afford the rearranged alcohol. If the ring opening rate is known (17), the rebound rate, $k_{\text{oxidation}}$, can be calculated according to Equation 2.

$$k_{\text{oxidation}} = k_{\text{ring opening}}(\text{unrearranged alcohol} / \text{rearranged alcohol}) \quad (\text{Eq. 2})$$

The hypothesis that substrate radical intermediates form in the sMMO system has been tested by a variety of techniques. Nitroxyl spin traps detected radical species produced during the oxidation of methane, methanol, and acetonitrile (22). The respective $\cdot\text{CH}_3$, $\cdot\text{CH}_2\text{OH}$, and $\cdot\text{CH}_2\text{CN}$ radicals apparently were able to diffuse out of the protein and react with the spin traps. The products were not quantitated, however, so it is difficult to gauge the importance of these species in the bulk mechanism. Early radical clock results employing sMMO from *Methylosinus trichosporium* OB3b suggested both radical and cationic intermediates (23). Later radical clock studies revealed no radical rearrangement for the *M. capsulatus* (Bath) system and very little for the enzyme from *M. trichosporium* OB3b (19, 24). Hydroxylations of chiral tritiated alkanes proceeded with ~30% inversion of stereochemistry, an amount corresponding to putative radical lifetimes (<100 fs) too short to be attributed to formation of a discrete radical intermediate (25, 26).

The possibility exists that substrate-dependent reactivity might invalidate the conclusions of research based on diagnostic substrate probes. In recent work,³ we obtained evidence that an intermediate other than Q, the active high valent diiron species that effects methane hydroxylation (1–3), is competent to oxidize olefins. These results demonstrated that substrate can indeed influence the reaction pathway. Conversely, it is also possible that the protein, through steric constraints or other interactions, might alter the reactivity of the probe from that observed free in solution. If ring opening were impeded, the clocks would "run slow" in the enzyme active site. These

³ Valentine, A. M., Stahl, S. S., and Lippard, S. J. (1999) *J. Am. Chem. Soc.*, in press.

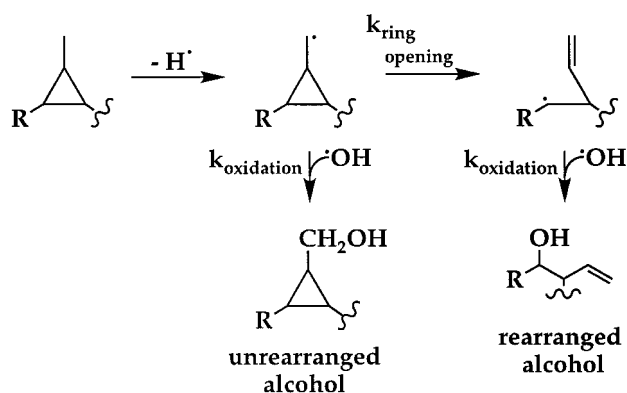


FIG. 1. Ring opening of cyclopropylcarbinyl radicals resulting from hydrogen atom abstraction from a radical clock substrate probe.

concerns (24) have been addressed by using probes that do not dramatically change shape on rearrangement. The best examples of such behavior are the round trip probes (19). A radical formed by H atom abstraction from the methyl group of methylcubane, for example, ends up on the same carbon atom, so there is no need for substrate translocation in the active site before rebound. The oxidation of methylcubane by sMMO from *M. capsulatus* (Bath) yielded no rearranged products (19).

The results of the radical clock substrate probe studies have thus far been in agreement with those using chiral tritiated alkanes (25, 26), the closest substrates to methane from which mechanistic information can be obtained. Nevertheless, we were interested in trying recently developed hypersensitive radical clock substrate probes (Table I), which have ring opening rates in excess of $10^{11}/s$. Such values allow detection of rebound reactions occurring with rate constants up to $10^{13}/s$, assuming a detection limit of 1% of ring-opened *versus* total product. This rate constant is at the limit for a barrier-less decomposition of a transition state (26). Probe 1 (24) can be prepared in pure (*R,R*) and (*S,S*) form. Probe 2 is constrained to require minimal conformational change upon ring opening (28). Probes 3 and 4 are substituted at the *para* position on the phenyl ring, eliminating the presence of "uninformative" phenol products (species 1b). These substitutions also increase the ring-opening rate constant. Probe 5 discriminates between radical and carbocationic intermediates (Fig. 2 and Ref. 18). Here we report the results of oxidation of these substrate probes by the reconstituted sMMO catalytic system. These and other data are used to assess some recent mechanistic proposals for catalysis.

EXPERIMENTAL PROCEDURES

Protein Preparation and Characterization—Native MMOH (24, 29, 30) and MMOR (26) and recombinant MMOB (24)⁴ were purified and characterized as previously reported. The specific activity of MMOH was in the range of 200–300 milliunits/mg in a standard propylene oxidation assay (24, 30).

Radical Clock Substrate Probes and Products—Probes and authentic samples of possible oxidation products were prepared as undiluted liquids or solids. The methods have been reported for probe 1 and its resolved enantiomers (32) 2 (33), 4 (34), and 5 (18). Probe 3 was prepared by similar methods, the details of which will be reported.^{5,6}

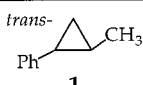
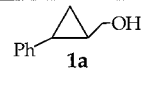
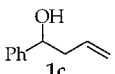

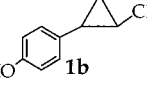
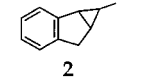
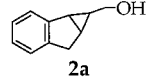
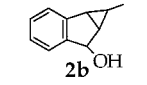
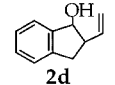
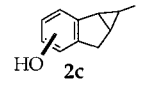
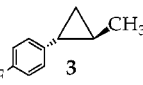
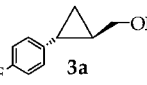
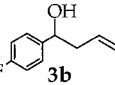
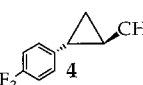
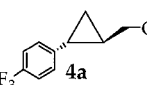
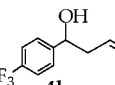
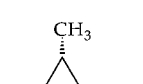
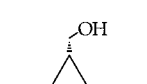
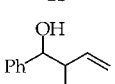
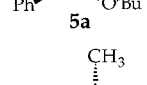
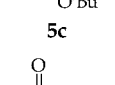
Kinetics of Radical Ring Openings—Rate constants for rearrangements of the putative radical intermediates were reported for the cyclopropylcarbinyl radicals derived from probes 1 (36), 2 (28), and 5 (37)

⁴ D. E. Coufal, J. Blazyk, D. A. Whittington, and S. J. Lippard, manuscript in preparation.

⁵ Newcomb, M., Choi, S.-Y., and Toy, P. (1999) *Can. J. Chem.*, in press.

⁶ Newcomb, M., Choi, S.-Y., and Horner, J. H. (1999) *J. Org. Chem.*, in press.

TABLE I
Radical clock probes and products used in the current study

Substrate	Unrearranged Products	Rearranged Product(s)
		
		
		
		
		
		
		
		
		

(Table II). The rate constants for rearrangement of the cyclopropylcarbinyl radicals derived from probes 3 and 4 were determined by competitive PhSeH trapping.^{5,6} All rate constants quoted in this work reflect a recent recalibration of the rate constants for reaction of PhSeH with alkyl radicals, which resulted in a reduction in the derived rate constants for ring opening by ~30%.

Enzymatic Oxidations and Product Characterization—Typically, 15 nmol of MMOH, 15 nmol of MMOB, and 5.25 nmol of MMOR were combined in a 20-ml glass scintillation vial. Probes 1–4 were added as undiluted liquids, usually as 1- μ l aliquots. Probe 5 was added as 2 ml of a saturated solution in 25 mM 4-morpholinepropanesulfonic acid (pH 7). The solution was incubated for 2 min at 45 °C. A 100- μ l aliquot of 0.1 M ethanol-free NADH was added to initiate the reaction and to bring the total volume to 5 ml, and the reaction was allowed to proceed at 45 °C for 15 min with gentle agitation. The mixture was extracted with methylene chloride (5×4 ml), and the solution was spun in an analytical centrifuge for 10 min after each extraction. The combined extracts (20 ml) were dried over $MgSO_4$ and concentrated to ~100 μ l by using a rotary evaporator. A 10- μ l aliquot of a 10 mM solution of an internal standard, methyl benzoate or ethyl benzoate, was added. The products were analyzed by parallel gas chromatography and gas chromatography with mass spectrometry methods on 50 m HP-1 methyl silicone columns. A Hewlett-Packard 5890A gas chromatograph equipped with a Hewlett-Packard 5971A mass spectrometer was used. Standard curves were generated by analyzing a range of concentrations of each substrate and product referenced to the same internal standard added to the reaction mixture. To determine whether products were consumed during the reaction or lost during workup, reaction solutions were spiked with small known amounts of these species, and the resulting product mixtures were analyzed for their presence. No such loss or consumption was observed.

To test for possible enzyme inhibition by probe 5, the reconstituted protein system was incubated with NADH and this probe for 5 min

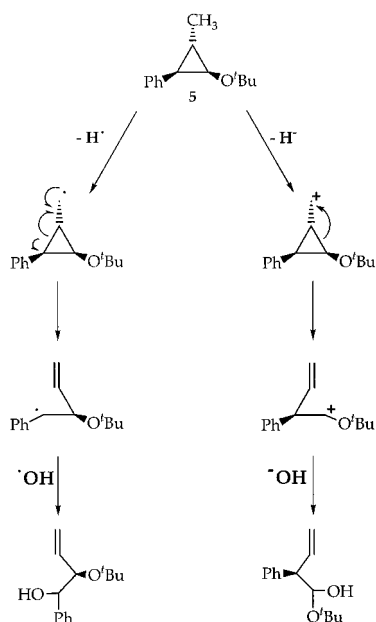


FIG. 2. **Ring opening of probe 5.** This probe was designed to discriminate between radical and cationic intermediates.

TABLE II
Rate constants for radical ring openings^a

Radical from	Arrhenius function ^b	<i>k</i>		
		25 °C	37 °C	45 °C
		<i>s</i> ⁻¹		
1	log <i>k</i> = 13.80 - 3.46/ <i>θ</i>	1.8 × 10 ¹¹	2.3 × 10 ¹¹	2.6 × 10 ¹¹
2	log <i>k</i> = 13.66 - 3.35/ <i>θ</i>	1.6 × 10 ¹¹	2.0 × 10 ¹¹	2.3 × 10 ¹¹
3		1.5 × 10 ¹¹		
4	log <i>k</i> = 13.43 - 2.34/ <i>θ</i>	4.2 × 10 ¹¹	6.0 × 10 ¹¹	6.6 × 10 ¹¹
5	log <i>k</i> = 14.24 - 3.87/ <i>θ</i>	2.5 × 10 ¹¹	3.2 × 10 ¹¹	3.8 × 10 ¹¹

^a Corrected for recent PhSeH recalibration.

^b *θ* = 2.3(*RT*) (in kcal/mol).

before addition of a second substrate. In some cases, probe **5** was removed by transferring the solution after preincubation to Centriplus 100 centrifugal concentrators (Amicon) and repeatedly concentrating and diluting it with 25 mM 4-morpholinepropanesulfonic acid (pH 7). MMOB, MMOR, NADH, and free probe **5** were removed by this procedure. The concentration of the resulting hydroxylase solution was determined and the absence of NADH was established by UV/visible spectroscopy. Removal of the other proteins was verified by SDS-polyacrylamide gel electrophoresis. The system was reconstituted with MMOB and MMOR, substrate, and NADH, and oxidation of a second probe was attempted as described above for radical clock probe substrates or by standard methods when propylene or nitrobenzene was used (24).

RESULTS AND DISCUSSION

Characterization of Oxidation Products—The oxidation products derived from the radical clock substrate probes are shown in Table III. Typically, <10% of the substrate was oxidized, and >85% of added material was recovered as products or unreacted probe. None of the products was consumed during the course of the reaction. In no case was a rearranged or ring-opened product detected. Less conversion was observed for probes **3** and **4** than for the unfluorinated analog probe **1**, probably because of their lower solubility. Only primary alcohol products were observed for these substrates.

In previous work reporting hydroxylation of racemic probe **1**, there was a slight preference for forming the primary alcohol over the phenol (24). It was suggested that this preference might reflect the conformation of the substrate molecule, such that one enantiomer might afford predominantly phenol and the other the primary alcohol. A comparison of the hydroxyla-

TABLE III
Products from oxidation of radical clock substrate probes by sMMO

Probe	Relative product yields ^a	Average conversion
		%
1	1a:1b:1c 1.1:1:0	6.4
2	2a:2b:2c:2d 0.48:0.12:1:0	10.5
3	3a:3b 1:0	3.8
4	4a:4b 1:0	2.5
5		

^a See Table I.

tion products of (*S,S*)- and (*R,R*)-1-phenyl-2-methylcyclopropane (Table IV) reveals that, when (*R,R*)-1 was used as substrate, the primary alcohol was formed preferentially. When the (*S,S*) enantiomer was used, the phenol was predominantly formed. This result supports the hypothesis that each enantiomer has a favored orientation within the hydrophobic cavity at the MMOH active site.

To understand these stereochemical preferences, a model of the enzyme active site was generated by using the 1.7-Å resolution MMOH x-ray crystallographic coordinates (38). The (*R,R*) and (*S,S*) enantiomers were docked into the active site, positioning either the methyl or phenyl group near the iron atoms and extending the remainder of the molecule into the adjacent hydrophobic cavity 1 (6). When the *para*-carbon atom of the phenyl ring for either substrate was positioned near the iron atoms, steric interactions were minimized by directing the methylcyclopropyl group out toward the neighboring hydrophobic cavity, cavity 2, in the vicinity of a mobile leucine residue, Leu-110 (Fig. 3, A and B, and Ref. 39). Attempts to position the phenyl ring near this leucine residue were unfavorable and did not orient the methyl group of the substrate near coordination sites on the diiron center previously identified as those most likely to generate activated oxygen species (40). Instead, the phenyl group was more readily accommodated when positioned toward helices E and F, which help form the canyon walls (6) of MMOH. When the substrate was so oriented, the methyl group of the (*R,R*) enantiomer was positioned directly over the diiron center (Fig. 3C), whereas the methyl group of the (*S,S*) enantiomer (Fig. 3D) was directed away from it. If the substrate does indeed bind in these two alternative modes, this model explains the more favorable oxidation of the methyl compared with the phenyl substituent observed for the (*R,R*) probe. The internal packing differences for the (*S,S*) substrate are less disparate, however, and might reflect protein side chain movements induced by MMOB binding, not observed in the structure of oxidized MMOH alone.

Attempts to oxidize probe **5** failed to yield any product alcohol, a result that suggested that this molecule might be a mechanism-based inhibitor. Probe **5** did inhibit the ability of the sMMO system to oxidize probe **1**, the largest of the substrates used, and this inhibition could be reversed by removal of the probe. Propylene and nitrobenzene oxidation were not affected by the presence of probe **5**, however. Thus, probe **5** does not covalently modify the protein but may be difficult to dislodge from the series of hydrophobic cavities leading to the enzyme active site cavity, which define a proposed pathway for substrate access (6, 38, 40). Several such cavities have been identified in the MMOH structure, and the path taken by a given substrate may depend on its steric requirements. The fact that activity toward small substrates is unaffected suggests that probe **5** cannot access the enzyme active site, explaining the observed lack of activity for this probe. Probe **5** did not fit well when modeled into the enzyme active site (Fig. 4).

Attempts to bring either the methyl or phenyl group near the iron center resulted in steric clashes between the *t*-butoxy group and protein side chains. The orientation shown, with the *t*-butoxy group pointing toward the iron center, was the most favorable but still would require significant protein side chain movements.

Calculation of a Lower Limit for the Rebound Rate Constant of a Putative Radical Intermediate—Detection of any rearranged products would have provided evidence for the intermediacy of a substrate radical. In the absence of such products, Equation 2 can be used to calculate the lower limit on the rate constant such a process would require to occur without ring

opening. Assuming a 10% detection limit for rearranged *versus* unrearranged product in the oxidation of probe 4, the fastest probe tested, the corresponding rebound rate would be $>7 \times 10^{12}/s$, and the lifetime of the putative radical would be at most 150 fs. These results are completely consistent with our findings for sMMO from *M. capsulatus* (Bath) with radical clock substrate probes (24) and chiral-tritiated alkanes (26). It should be noted that the apparent lifetimes appear to be slightly different for *M. trichosporium* (OB3b) sMMO, as revealed by the chiral ethane and butane oxidations (25, 26) and the radical clock substrate probes (24). None of the values determined for either enzyme, however, is consistent with formation of a discrete substrate radical. Thus the present results strongly reinforce our previous conclusions that a radical rebound mechanism does not occur for sMMO and that a non-synchronous, concerted process better explains the experimental data (18, 26, 41). Some possible oxidation mechanisms are depicted in Fig. 5 and discussed in more detail below.

Mechanistic Implications and Comparisons with Literature Results—Many possible mechanisms for the catalytic cycle in MMOH have been compiled in review and opinion articles regarding sMMO (2, 24, 42). These mechanisms are supported in part by the detection of intermediate species in single turnover studies of the enzyme (10, 43–45). The oxidation step typically involves reaction of an electrophilic intermediate Q, postulated to be a bis(μ -oxo)diiron(IV) (46) or related species, with the hydrocarbon substrate. Some agreement has been

TABLE IV
Products from oxidation of racemic *trans*-1-phenyl-2-methylcyclopropane and of each enantiomer

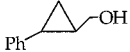
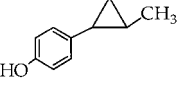
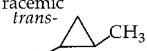

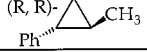
Substrate		
racemic <i>trans</i> - 	1.1	1
(<i>S,S</i>)- 	0.70	1
(<i>R,R</i>)- 	1.36	1

FIG. 3. Binding of probe 1 in the MMOH active site with the phenyl ring (A and B) or the methyl group (C and D) pointing toward the presumed location of the activated oxygen species. A and C, (*R,R*) enantiomer. B and D, (*S,S*) enantiomer.

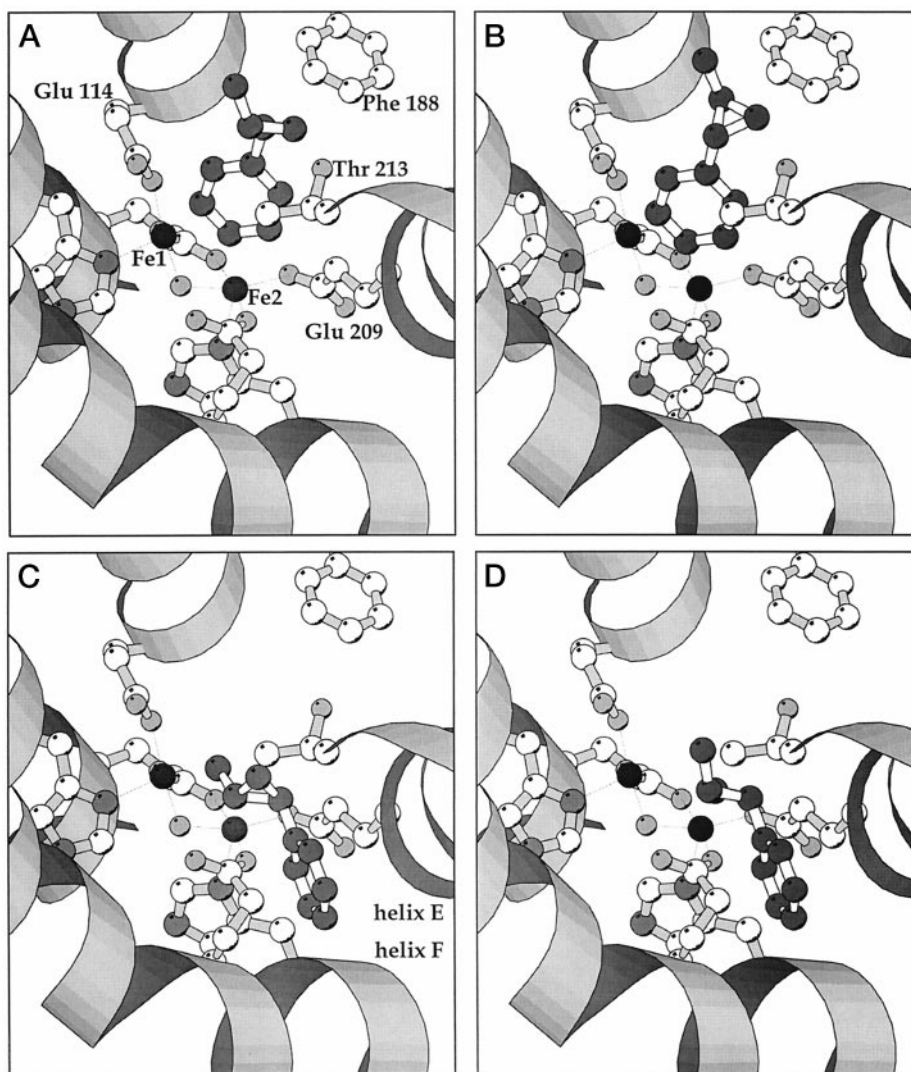




FIG. 4. Binding of probe 5 in the MMOH active site.

reached that freely diffusing substrate radicals are not a hallmark of sMMO oxidation chemistry, although some workers still draw a discrete radical intermediate (R) (47, 48). How best to explain the available mechanistic information remains a contested topic.

Recently, a set of commentaries appeared that speculated on the hydrocarbon oxidation mechanism (49). Some common questions that emerged are the following: What is the structure of Q ? Is Q the active species? Is the oxygen transferred to substrate bridging the iron atoms (50) or terminal (11, 51) in the active species? Are Fe–C bonds formed and, if so, before (27, 31, 53) or after (11, 47, 48, 51) C–H bond cleavage (41)?

Some favor a ferryl species for Q as the active intermediate (47), with concerted addition of the C–H bond across the Fe(IV)=O unit and some component of Fe–C bonding. Radical traps are claimed to be able to intercept the C–OH bond as it forms in the transition state. The presence of terminal Fe=O bonds in Q remains a viable possibility, although it is not supported by extended x-ray absorption fine structure spectroscopy (46) and modeling chemistry (35). Perhaps Q is in dynamic equilibrium with such a species, as we have previously suggested (3). The lack of quantitation in the radical trap experiments has already been noted. In recent work (52), inhibition of cyclohexane oxidation by H_2 , D_2 , and CH_4 provided evidence for an agostic interaction of these molecules involving a porphyrin Fe(IV)=O unit. Both steady state⁷ and single turnover⁸ sMMO oxidations are unaffected by the presence of H_2 , as would be expected if such an interaction were taking place.

Other workers (50) prefer a “bridge mechanism,” or oxidation by a bis(μ -oxo)diiron(IV) active species. Such an intermediate structure has been combined with the above oxidation mechanism in an extended Hückel theoretical analysis of the oxidation of methane by a ferryl species (53). When applied to sMMO (31), drawn for the general substrate R–H in Fig. 5, *upper pathway*, methane undergoes a C_{3v} distortion and coordinates to the iron atom of a bis(μ -oxo)diiron(IV) active species. The resulting five-coordinate carbon could undergo pseudorotation, which was one of the explanations offered to account for the chiral ethane results (26). This process would invert the chiral ethane molecule without forming a discrete radical. A hydrogen

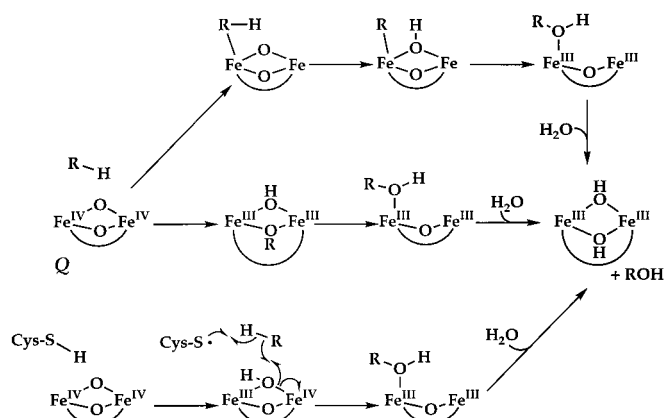


FIG. 5. Possible mechanisms for the oxidation of substrates by sMMO.

shift to the bridging oxygen atom followed by a methyl shift would yield the product complex (29).

Still other investigators bring to the debate a combination of computational and structural insights (11, 51). According to density functional theory, the active intermediate is an Fe(III)–O–Fe(IV)–O species, which reacts by hydrogen atom abstraction to afford a caged radical. Rapid formation of an Fe(V)–C bond ensues. The very short lifetime limit determined in the radical clock and chiral ethane experiments would correspond to the period between hydrogen atom abstraction and iron–carbon bond formation. These translational motions could not take place in the <150-fs limit set by our current results, however. The authors postulate that the Fe–C bond could be very weak and that the alkyl could still be intercepted by spin traps, but again the time scales seem incommensurate. Still others (48) support an oxo-bridged Q structure, which generates an intermediate having radical character, perhaps according to one of the mechanisms described above, but without forming discrete free radicals.

The current proposals for the structure of the oxidizing intermediate thus fall into three classes: a bis(μ -oxo)diiron(IV), a diferryl, and a Fe(III)–O–Fe(IV)–O species. Iron–carbon bonds are usually invoked, either before or after C–H bond cleavage, despite the fact that there is so far no experimental evidence to support them. The reaction itself must proceed by some version of a nonsynchronous concerted pathway to allow partial inversion of chiral ethane without generating free substrate radicals. One such mechanism depicting oxidation by Q is given in the middle pathway of Fig. 5. This mechanism is similar to that proposed elsewhere, except that the active species drawn was a diferryl rather than a bridged structure (26). In this model, reaction of substrate with the active species affords hydroxide and alkoxide, which are protonated to release the products.

In Fig. 5, *bottom*, is a mechanism that invokes participation of the nearby cysteine sulfhydryl group. Its involvement has been proposed previously (42). In this mechanism, hydrogen atom transfer from the cysteine to Q affords a mixed valent, hydroxo-bridged species. This species would have to be very short-lived, because no such compound has been detected in rapid freeze quench Mössbauer time course studies without substrate or with methane. In a concerted reaction, the cysteine radical and iron center would cleave the C–H bond in a nonsynchronous fashion, during which time some radical character in the substrate might develop without formation of a discrete substrate radical species. Alternatively, if either bridging (or terminal) oxygen atom were able to attack the substrate, hydroxylation would occur on one face or the other of the RCH_3 molecule, accounting for the partial inversion of the chiral ethane probe.

⁷ A. M. Valentine and S. J. Lippard, unpublished results.

⁸ S. S. Stahl, and S. J. Lippard, unpublished results.

Conclusions—Results from oxidations of hypersensitive radical clock substrate probes continue to refute the notion that a discrete substrate radical species forms in sMMO oxidations. For probe 1, which can be prepared in enantiomerically pure form, the predominant oxidation product depends on the enantiomer used, providing evidence that the substrate assumes a preferred binding mode in the enzyme active site. Although probe 5 is not oxidized by sMMO, it inhibits oxidation of probe 1 but not propylene or nitrobenzene. This inhibition is reversed after removal of probe 5, suggesting that it sterically blocks access to the active site for the large substrate probe 1. Several recent mechanistic proposals incorporate aspects of a nonsynchronous, concerted mechanism and attempt to reconcile all the existing spectroscopic and mechanistic data regarding sMMO. Additional mechanistic, experimental, and theoretical work is clearly warranted.

Acknowledgment—We thank D. A. Whittington for help with preparation of Figs. 4 and 5.

REFERENCES

- Liu, K. E., and Lippard, S. J. (1995) in *Advances in Inorganic Chemistry* (Sykes, A. G., ed) Vol. 42, pp. 263–289, Academic Press, San Diego
- Wallar, B. J., and Lipscomb, J. D. (1996) *Chem. Rev.* **96**, 2625–2657
- Valentine, A. M., and Lippard, S. J. (1997) *J. Chem. Soc. Dalton Trans.* **21**, 3925–3931
- Colby, J., and Dalton, H. (1978) *Biochem. J.* **171**, 461–468
- Woodland, M. P., and Dalton, H. (1984) *J. Biol. Chem.* **259**, 53–59
- Rosenzweig, A. C., Frederick, C. A., Lippard, S. J., and Nordlund, P. (1993) *Nature* **366**, 537–543
- Rosenzweig, A. C., Nordlund, P., Takahara, P. M., Frederick, C. A., and Lippard, S. J. (1995) *Chem. Biol.* **2**, 409–418
- Colby, J., and Dalton, H. (1979) *Biochem. J.* **177**, 903–908
- Green, J., and Dalton, H. (1985) *J. Biol. Chem.* **260**, 15795–15801
- Liu, K. E., Valentine, A. M., Wang, D., Huynh, B. H., Edmondson, D. E., Salifoglou, A., and Lippard, S. J. (1995) *J. Am. Chem. Soc.* **117**, 10174–10185
- Siegbahn, P. E. M., Crabtree, R. H., and Nordlund, P. (1998) *J. Biol. Inorg. Chem.* **3**, 314–317
- Colby, J., Stirling, D. I., and Dalton, H. (1977) *Biochem. J.* **165**, 395–402
- Green, J., and Dalton, H. (1989) *J. Biol. Chem.* **264**, 17698–17703
- Dalton, H. (1980) in *Advances in Applied Microbiology*, Vol. 26, pp. 71–87, Academic Press
- Fox, B. G., Borneman, J. G., Wackett, L. P., and Lipscomb, J. D. (1990) *Biochemistry* **29**, 6419–6427
- Griller, D., and Ingold, K. U. (1980) *Acc. Chem. Res.* **13**, 317–323
- Newcomb, M. (1993) *Tetrahedron* **49**, 1151–1176
- Newcomb, M., Le Tadic-Biadatti, M.-H., Chestney, D. L., Roberts, E. S., and Hollenberg, P. F. (1995) *J. Am. Chem. Soc.* **117**, 12085–12091
- Choi, S.-Y., Eaton, P. E., Hollenberg, P. F., Liu, K. E., Lippard, S. J., Newcomb, M., Putt, D. A., Upadhyaya, S. P., and Xiong, Y. (1996) *J. Am. Chem. Soc.* **118**, 6547–6555
- Castellino, A. J., and Bruce, T. C. (1988) *J. Am. Chem. Soc.* **110**, 7512–7519
- Vanni, R., Garden, S. J., Banks, J. T., and Ingold, K. U. (1995) *Tetrahedron Lett.* **36**, 7999–8002
- Deighton, N., Podmore, I. D., Symons, M. C. R., Wilkins, P. C., and Dalton, H. (1991) *J. Chem. Soc. Chem. Commun.* **91**, 1086–1088
- Ruzicka, F., Huang, D.-S., Donnelly, M. I., and Frey, P. A. (1990) *Biochemistry* **29**, 1696–1700
- Liu, K. E., Johnson, C. C., Newcomb, M., and Lippard, S. J. (1993) *J. Am. Chem. Soc.* **115**, 939–947
- Priestley, N. D., Floss, H. G., Froland, W. A., Lipscomb, J. D., Williams, P. G., and Morimoto, H. (1992) *J. Am. Chem. Soc.* **114**, 7561–7562
- Valentine, A. M., Wilkinson, B., Liu, K. E., Komar-Panicucci, S., Priestley, N. D., Williams, P. G., Morimoto, H., Floss, H. G., and Lippard, S. J. (1997) *J. Am. Chem. Soc.* **119**, 1818–1827
- Yoshizawa, K., Shiota, Y., and Yamabe, T. (1997) *Chem. Eur. J.* **3**, 1160–1169
- Martin-Esker, A. A., Johnson, C. C., Horner, J. H., and Newcomb, M. (1994) *J. Am. Chem. Soc.* **116**, 9174–9181
- Willems, J.-P., Valentine, A. M., Gurbiel, R., Lippard, S. J., and Hoffman, B. M. (1998) *J. Am. Chem. Soc.* **120**, 9410–9416
- DeWitt, J. G., Bentsen, J. G., Rosenzweig, A. C., Hedman, B., Green, J., Pilkington, S., Papaefthymiou, G. C., Dalton, H., Hodgson, K. O., and Lippard, S. J. (1991) *J. Am. Chem. Soc.* **113**, 9219–9235
- Yoshizawa, K. (1998) *J. Biol. Inorg. Chem.* **3**, 318–324
- Atkinson, J. K., Hollenberg, P. F., Ingold, K. U., Johnson, C. C., Le Tadic, M.-H., Newcomb, M., and Putt, D. A. (1994) *Biochemistry* **33**, 10630–10637
- Newcomb, M., Le Tadic, M.-H., Putt, D. A., and Hollenberg, P. F. (1995) *J. Am. Chem. Soc.* **117**, 3312–3313
- Toy, P. H., Dhanabalasingam, B., Newcomb, M., Hanna, I. H., and Hollenberg, P. F. (1997) *J. Org. Chem.* **62**, 9114–9122
- Lee, D., and Lippard, S. J. (1998) *J. Am. Chem. Soc.* **120**, 12153–12154
- Newcomb, M., Johnson, C. C., Manek, M. B., and Varick, T. R. (1992) *J. Am. Chem. Soc.* **114**, 10915–10921
- Le Tadic-Biadatti, M.-H., and Newcomb, M. (1996) *J. Chem. Soc. Perkin Trans. 2*, 1467–1473
- Rosenzweig, A. C., and Lippard, S. J. (1994) *Acc. Chem. Res.* **27**, 229–236
- Rosenzweig, A. C., Brandstetter, H., Whittington, D. A., Nordlund, P., Lippard, S. J., and Frederick, C. A. (1997) *Proteins* **29**, 141–152
- Whittington, D. A., and Lippard, S. J. (1998) in *ACS Symposium Series* (Hodgson, K. O., and Solomon, E. I., eds) **692**, 334–347
- Whittington, D. A., Valentine, A. M., and Lippard, S. J. (1998) *J. Biol. Inorg. Chem.* **3**, 307–313
- Feig, A. L., and Lippard, S. J. (1994) *Chem. Rev.* **94**, 759–805
- Lee, S.-K., Fox, B. G., Froland, W. A., Lipscomb, J. D., and Münck, E. (1993) *J. Am. Chem. Soc.* **115**, 6450–6451
- Lee, S.-K., Nesheim, J. C., and Lipscomb, J. D. (1993) *J. Biol. Chem.* **268**, 21569–21577
- Liu, K. E., Wang, D., Huynh, B. H., Edmondson, D. E., Salifoglou, A., and Lippard, S. J. (1994) *J. Am. Chem. Soc.* **116**, 7465–7466
- Shu, L., Nesheim, J. C., Kauffmann, K., Münck, E., Lipscomb, J. D., and Que, L., Jr. (1997) *Science* **275**, 515–518
- Deeth, R. J., and Dalton, H. (1998) *J. Biol. Inorg. Chem.* **3**, 302–306
- Lipscomb, J. D., and Que, L. J. (1998) *J. Biol. Inorg. Chem.* **3**, 331–336
- Nordlander, E., and Andersson, K. K. (1998) *J. Biol. Inorg. Chem.* **3**, 300–301
- Shteinman, A. A. (1998) *J. Biol. Inorg. Chem.* **3**, 325–330
- Siegbahn, P. E. M., and Crabtree, R. H. (1997) *J. Am. Chem. Soc.* **119**, 3103–3113
- Collman, J. P., Chien, A. S., Eberspacher, T. A., and Brauman, J. I. (1998) *J. Am. Chem. Soc.* **120**, 425–426
- Yoshizawa, K., Shiota, Y., and Yamabe, T. (1998) *Organometallics* **17**, 2825–2831