Regio- and Stereoselectivity of Particulate Methane Monooxygenase from *Methylococcus capsulatus* (Bath)

Sean J. Elliott, Mei Zhu, Luke Tso, H.-Hoa T. Nguyen, John H.-K. Yip, and Sunney I. Chan*

Contribution from the A. A. Noyes Laboratory of Chemical Physics, 127-72, California Institute of Technology, Pasadena, California 91125

Received April 3, 1997[⊗]

Abstract: The regiospecificity and stereoselectivity of alkane hydroxylation and alkene epoxidation by the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath) was evaluated over a range of substrates. Oxidation products were identified by conventional GC analysis, and the stereoselectivity of oxidation was determined by a combination of chiral GC and HPLC methods, as well as ^{1}H NMR analysis of the corresponding (*R*)-2-acetoxy-2-phenylethanoate ester derivatives in the case of alkanol products. Alkane hydroxylation was found to proceed favoring attack at the C-2 position in all cases, and the stereoselectivity for *n*-butane and *n*-pentane was characterized by an enantiomeric excess of 46% and 80%, respectively, with preference for the (*R*)-alcohol noted for both substrates. Epoxides were formed with smaller stereoselectivities. Together, the regio- and stereoselectivity results suggest that an equilibrium of competing substrate binding modes exists. A simple substrate-binding model that incorporates preferential C-2 oxidation with the observed stereoselectivity of alkane hydroxylation is proposed, and hypotheses for the general mechanism are suggested and discussed.

The particulate methane monooxygenase (pMMO) from *Methylococcus capsulatus* (Bath) catalyzes the dioxygen-dependent, two-electron oxidation of methane to methanol.[1−3] The enzyme is a copper-containing membrane protein comprised of three subunits of 47-, 23-, and 20-kDa molecular mass.[1,3] Similar to its soluble counterpart (sMMO), pMMO has garnered increased attention of late due to its ability to activate dioxygen and hydrocarbon C−H bonds. In cell cultures, the copper(II) content has been correlated to pMMO activity,[4] as well as the active repression of sMMO transcription.[5] The addition of copper(II) has been shown to stimulate pMMO activity in a cell-free membrane system, while other divalent transition metals such as nickel(II) and zinc(II) do not.[6] Further, in cell-free assays, the inhibition of pMMO activity by the suicide substrate acetylene has been correlated to the total concentration of copper(II) ions in the assay.[6] The active site of pMMO is surmised to contain a trimeric copper cluster,[1] whereas the sMMO active site contains a diiron, hydroxyl-bridged cluster.[7] The hydroxylase component of sMMO exists as an $\alpha_2\beta_2\gamma_2$ dimer which has been crystallized recently, and its three-dimensional structure has been determined by X-ray crystallography.[8,9] pMMO is expressed under copper-containing growth conditions in all known methanotrophs, while sMMO has been found only in a subset of methanotrophic organisms, and it is expressed in copper-free, iron-rich growth conditions only. Further, while sMMO has demonstrated a wide range of substrate specificity, including straight-chain, branched, and aromatic C−H bonds,[10−13] pMMO mediates hydroxylation and epoxidation for a small set of straight-chain alkane and alkene substrates solely.[3,14]

Determining the mechanism of C−H bond activation in biological systems is of great interest currently.[15,16] To this end, the mechanism of sMMO-mediated hydroxylation has been studied with radical spin-traps[17] as well as radical clock substrate probes.[18] Several mechanisms, which have been suggested to account for the observations, invoke alkyl free radical and/or carbocation structures as potential intermediate species[13,18−20] or a concerted process involving direct oxygen insertion into a substrate carbon−iron bond.[21,22] Unfortunately, while pMMO warrants similar consideration due to its pervasive presence in methanotrophs, analogous studies using pMMO have been hindered by difficulty in maintaining the activity of the purified pMMO, as well as the limited substrate range of the enzyme.[1] However, the hydroxylation mechanisms of sMMO from *Methylosinus trichosporium* (OB3b) and pMMO from *M. capsulatus* (Bath) have recently been investigated by the use of cryptically chiral ethanes.[19,23,24] The determination of the

[⊗] Abstract published in *Advance ACS Abstracts,* October 1, 1997.

(1) Nguyen, H.-H. T.; Shiemke, A. K.; Jacobs, S. J.; Hales, B. J.; Lidstrom, M. E.; Chan, S. I. *J. Biol. Chem.* **1994**, *269*, 14995.
(2) Bédard, C.; Knowles, R. *Microbiol. Rev.* **1989**, *53*, 68.
(3) Nguyen, H.-H. N. T.; Zhu, M.; Elliott, S. J.; Nakagawa, K. H.; Hedman, B.; Costello, A. M.; Peeples, T. L.; Wilkinson, B.; Morimoto, H.; Williams, P. G.; Floss, H. G.; Lidstrom, M. E.; Hodgson, K. O.; Chan, S. I. In *Microbial Growth on C_1 Compounds*; Lidstrom, M. E., Tabita, F. R., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1996; p 150.
(4) Semrau, J. D.; Zolandz, D.; Lidstrom, M. E.; Chan, S. I. *J. Inorg. Biochem.* **1995**, *58*, 235.
(5) Nielsen, A. K.; Gerdes, K.; Degn, H.; Murrell, J. C. *Microbiology (Reading, UK)* **1996**, *142*, 1289.
(6) Cook, S. A.; Shiemke, A. K. *J. Inorg. Biochem.* **1996**, *63*, 273.
(7) Lipscomb, J. D. *Annu. Rev. Microbiol.* **1994**, *48*, 371.
(8) Rosenzweig, A. C.; Frederick, C. A.; Lippard, S. J.; Nordlund, P. *Nature* **1993**, *366*, 537.
(9) Rosenzweig, A. C.; Nordlund, P.; Takahara, P. M.; Frederick, C. A.; Lippard, S. J. *Chem. Biol.* **1995**, *2*, 409.
(10) Colby, J.; Stirling, D. I.; Dalton, H. *Biochem. J.* **1977**, *165*, 395.
(11) Stirling, D. I.; Dalton, H. *J. Gen. Microbiol.* **1980**, *116*, 277.
(12) Higgins, I. J.; Best, D. J.; Hammond, R. C. *Nature* **1980**, *286*, 561.
(13) Green, J.; Dalton, H. *J. Biol. Chem.* **1989**, *264*, 176980.
(14) Smith, D. D. S.; Dalton, H. *Eur. J. Biochem.* **1989**, *182*, 667.
(15) Feig, A. L.; Lippard, S. J. *Chem. Rev.* **1994**, *94*, 759.
(16) Shteinman, A. A. *FEBS Lett.* **1995**, *362*, 5.
(17) Dalton, H.; Wilkins, P. C.; Jiang, Y. In *Microbial Growth on C_1*; Murrell, J. C., Kelly, D. P., Eds.; Intercept: Andover, UK, 1993; p 65.
(18) Liu, K. E.; Johnson, C. C.; Newcomb, M.; Lippard, S. J. *J. Am. Chem. Soc.* **1993**, *115*, 939.
(19) Priestley, N. D.; Floss, H. G.; Froland, W. A.; Lipscomb, J. D.; Williams, P. G.; Morimoto, H. *J. Am. Chem. Soc.* **1992**, *114*, 7561.
(20) Ruzicka, F.; Huang, D.-S.; Donnelly, M. I.; Frey, P. A. *Biochemistry* **1990**, *29*, 1696.
(21) Barton, D. H. R.; Csuhai, E.; Doller, D.; Ozbalik, N.; Balavoine, G. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3401.
(22) Barton, D. H. R.; Bévière, S. D.; Chavasiri, W.; Csuhai, E.; Doller, D.; Liu, W.-G. *J. Am. Chem. Soc.* **1992**, *114*, 2147.

S0002-7863(97)01049-4 CCC: $14.00

Table 1. Hydroxylation and Epoxidation Product Chemistry Mediated by pMMO

substrate	products (% total)	k^{rel} [a]	dominant stereoisomer	ee (%)
ethane	ethanol	1.00[b]	na	
propane	propan-2-ol (~100)	0.90	na	
	propan-1-ol (trace)	nd	na	
n-butane	butan-2-ol (95)	0.4	*R*[c]	46 ± 4.5
	butan-1-ol (5)	0.02	na	
n-pentane	pentan-2-ol (95)	0.17	*R*[c]	80 ± 2
	pentan-1-ol (5)	<0.01	na	
2-methylpropane	none	na	na	
2-methylbutane	3-methylbutan-1-ol (trace)	nd	na	
propylene	propylene oxide	1.0[b]	*S*[d]	18.5 ± 2
1-butene	1-epoxybutane (58)	0.62	*S*[e]	37 ± 2
	3-buten-2-ol (42)	0.70[b]	*S*[e]	50 ± 4
1,3-butadiene	1,3-butadiene monoxide	0.96	*R*[e]	15 ± 2
cis-but-2-ene	2,3-*cis*-epoxybutane	0.29	na	
trans-but-2-ene	2,3-*trans*-epoxybutane	0.26	nd	

[a] Notes: na, not applicable; nd, not determined. [b] For alkane substrates, rates are relative to ethane hydroxylation; for alkene substrates, rates are relative to propylene epoxidation. [c] Relative to ethane hydroxylation. [c] Assigned by comparison to the (R)-2-acetoxy-2-phenylethanoate ester derivative of (R)-butan-2-ol by ^{1}H NMR. [d] The absolute configuration was assigned by GC analysis using a Chiraldex A-TA column (20 m × 0.25 mm) by comparison with authentic standards of (R)- and (S)-propylene oxide (Fluka). [e] The absolute configuration was assigned using a Chiraldex G-TA column, with comparison to (*R*)- and (*S*)-epichlorohydrin (Aldrich) and the trichloroacetic acid derivative of (*R*)- and (*S*)-glycidol (Aldrich) , assuming identical elution behavior of (*R*)- and (*S*)-isomers.

stereoselectivity in ethanol production using either (*R*)- or (*S*)-[1,1-^{3}H$_1$,^{2}H$_1$] ethane demonstrated essentially 100% stereoselection for pMMO and retention accompanied by substantial inversion of configuration for sMMO. In this light, a concerted reaction mechanism was proposed for pMMO-mediated hydroxylation of methane and ethane, with the further suggestion of a pentacoordinate carbon species serving as an intermediate.[23] Alternatively, sMMO-mediated hydroxylation was determined to proceed *via* a short-lived alkyl radical in the case of the *M. trichosporium* (OB3b) enzyme,[19] and it was decided that a concerted yet nonsynchronous mechanism that contains an extremely short-lived alkyl radical structure in the transition state is a more appropriate description for sMMO from *M. capsulatus* (Bath).[24,25] The mechanistic hypotheses associated with each of these systems are of great interest currently and have yet to be resolved unequivocally.

In light of our previous observation of the stereospecific oxidation of chiral ethanes by pMMO, we have proceeded to investigate the potential of pMMO for mediating regio- and stereoselective oxidations of other hydrocarbon substrates. In part, such a study focuses on the issue of the presentation of substrate molecules in the active site (regiochemistry); however, the results may well also shed additional insights into the nature of the previously suggested concerted mechanism (stereoselectivity.) Previously, investigators have studied the product distribution and regiospecificity of alkane and alkene oxidations mediated by MMOs from *M. capsulatus* (Bath) and *M. trichosporium* (OB3b), finding that both purified sMMO and sMMO-containing cell-free extracts oxidize a wide range of aliphatic and aromatic hydrocarbons, with little rigorous regiospecificity.[10−13] On the other hand, using the purified multicomponent sMMO system from *M. trichosporium* (OB3b), it has been shown that alteration of the ratio of regulatory and reductase components greatly alters the regiospecificity of hydroxylation for a given substrate.[26]

Here we present a study of the regiospecificity of pMMO-catalyzed alkane and alkene oxidation by utilizing a broader range of substrates than attempted heretofore. We also present additional nuances on the *stereo*specificity of these oxidation reactions.

Experimental Procedures

Enzyme-Mediated Oxidations. Cultures of *M. capsulatus* (Bath) were grown on a 10 L scale on nitrate mineral salts medium, and pMMO-containing membrane fractions from *M. capsulatus* (Bath) were isolated as described previously.[1] These membrane fractions were highly enriched in pMMO and provided an ideal source of enzyme and were used to prepare oxidation products from the substrates listed in Table 1. The products were generated by the incubation of 2.5 mL of a pMMO-containing membrane suspension (with a total protein concentration of 12−16 mg/mL and a specific activity of 10−15 nmol/(min·mg)) with 35 μmol of NADH (Sigma) in 25 mL of 50 mM PIPES buffer (pH 7.3) at 45 °C, as described previously.[23] The reactions were initiated by the injection of 10 mL of gaseous substrate, so that all reactions were carried out in the presence of excess substrate such that the reaction velocity was assumed to be at V_{max}. For the assays carried out to determine product distribution and relative rates of product formation, products were generated on a 1-mL scale, using 0.1 mL of membrane suspension, while the determination of the stereospecificity of oxidations was carried out on a 25-mL scale, using 2.5 mL of membranes, as described above.

Product Identification and Quantification. The identification of products, as well as the quantitation of products for the purpose of determining relative rates of formation, was achieved by the comparison of GC retention times using a Hewlett-Packard HP5840A gas chromatograph fitted with a 1% AT-1000/Carbograph 60/80-mesh column and a flame ionization detector. Primary and secondary alcohol standards, as well as epoxidation standards, obtained from Aldrich were used to identify and quantify the distribution of products. Aliquots of reaction products were taken aside at 2−4-min intervals for the first 20 min of the reaction and immediately placed at 0 °C, quenching the reaction. Subsequent aliquots were taken aside at 7-min intervals thereafter, monitoring the remaining time course of the reaction (usually <1 h). The aliquots were centrifuged briefly, pelleting the membranes, and 1 μL of the supernatant was applied directly to the GC. The integrated peaks from GC traces corresponding to products were then used to determine the respective rates of formation for each of the products under study. Each product was quantified by comparing the peak size to a calibration curve for each of the possible oxidation

(23) Wilkinson, B.; Zhu, M.; Priestley, N. D.; Nguyen, H.-H. T.; Morimoto, H.; Williams, P. G.; Chan, S. I.; Floss, H. G. *J. Am. Chem. Soc.* **1996**, *118*, 921.

(24) Valentine, A. M.; Wilkinson, B.; Liu, K. E.; Komar-Panicucci, S.; Priestley, N. D.; Williams, P. G.; Morimoto, H.; Floss, H. G.; Lippard, S. J. *J. Am. Chem. Soc.* **1997**, *119*, 1818.

(25) Choi, S.-Y.; Eaton, P. E.; Hollenber, P. F.; Liu, K. E.; Lippard, S. J.; Newcomb, M.; Putt, D. A.; Upadhyaya, S. P.; Xiong, Y. *J. Am. Chem. Soc* **1996**, *118*, 6547.

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

products. Only membrane preparations demonstrating a k_{obs} for ethane oxidation of 12−17 nmol of ethanol/(min·mg protein) and a k_{obs} for propylene oxidation of 10−15 nmol of propylene oxide/(min·mg of protein) were used in these experiments. Relative rates of formation of products are reported due to slight variation (∼10%) between the baseline activities of ethane/propylene oxidation between membrane preparations.

Stereochemical Assignments. For those experiments designed to determine stereoselectivity of product formation, the reaction products were extracted into ether from the reaction mixture, and the ethereal layer was dried over $MgSO_4$ and concentrated under a nitrogen stream while immersed in an ice−salt bath. The stereochemical identity of the hydroxylation products of the oxidation of butane and pentane was assigned by comparison of the 300-MHz ^{1}H NMR spectra of the (*R*)-2-acetoxy-2-phenylethanoate ester derivatives with authentic standards, prepared from (*R*)- and (*S*)-butan-2-ol (Aldrich).[27] These data were confirmed by HPLC analysis of the derivatized products and standards using a D-DNB-leucine chiral column. Similarly, the absolute configuration of the epoxidation products was determined directly using either Chiraldex-ATA or Chiraldex-GTA GC-LC methodologies on a Hewlett-Packard 5890 Series II instrument, by comparing the products to the authentic standards (*R*)- and (*S*)-propylene oxide (Fluka), (*R*)- and (*S*)-epichlorohydrin (Fluka) and the trichloroacetic acid ester of (*R*)- and (*S*)-glycidol (Aldrich). Due to the commercial unavailability of enantiomerically pure standards for 1-epoxybutane and 1,3-butadiene monoxide, we used the epichlorohydrin- and glycidol-based standards as product analogs, which yielded consistent enantiomeric resolution, and assumed analogous elution behavior of the (*R*)- and (*S*)-isomers. A Chiraldex-ATA column (20 m × 0.25 mm) was used for analysis of propylene oxide formed from the oxidation of propylene, while a Chiraldex-GTA column (20 m × 0.25 mm) was used to evaluate the stereochemical configuration of the 1-epoxybutane and 1,3-butadiene monoxide produced from 1-butene and 1,3-butadiene, respectively. The enantiomeric excess (ee) values are defined as $|(R - S)|/(R + S)$, where *R* and *S* are the total amounts of each stereoisomer as determined by GC peak integration.

Results and Discussion

Regioselectivity of Hydrocarbon Oxidation. In our hands, pMMO-mediated oxidations of alkanes and alkenes are limited to straight-chain hydrocarbons containing five carbon atoms or less (Table 1). No oxidation products were detected for any substrate containing more than five carbon atoms. Further, branched hydrocarbons are not oxidized by pMMO, with a sole exception being 2-methylbutane, which yielded 3-methylbutan-1-ol in trace quantities. Substrates containing conformation-limiting double bonds, such as *cis*- or *trans*-2-butene, were oxidized exclusively to the corresponding epoxide, indicating that a loss in the conformational flexibility of a substrate does not terminate catalysis. Remarkably, oxidation was overwhelmingly favored in the C-2 position, *e.g.*, propane was oxidized to propan-2-ol solely, strongly contrasting with the wide-ranging regiospecificity of sMMO[10−13] and unlike a previous account of pMMO-mediated propan-1-ol and propan-2-ol formation.[14] Further, we found that oxidation of *cis*- and *trans*-2-butene yielded an epoxidation product only, and not the hydroxylation product crotyl alcohol or the further oxidation product, crotonaldehyde, as has been reported elsewhere.[14]

Stereoselectivity of Hydrocarbon Oxidation. Earlier studies of the stereoselectivity of pMMO using cryptically chiral ethanes revealed ∼100% retention of configuration in the hydroxylation products formed,[23] suggesting that a single stereoisomer should result with the other alkane substrates used in this study. However, while the stereoselectivity of the hydroxylation chemistry reported in Table 1 strongly favors the formation of (*R*)-alcohols, the chemistry is not 100% stereoselective. The observed ee values for the oxidation of *n*-butane and *n*-pentane vary significantly (46% and 80%, respectively), indicating that subtle steric constraints must be very important in determining the stereoselectivity (*vide infra*).

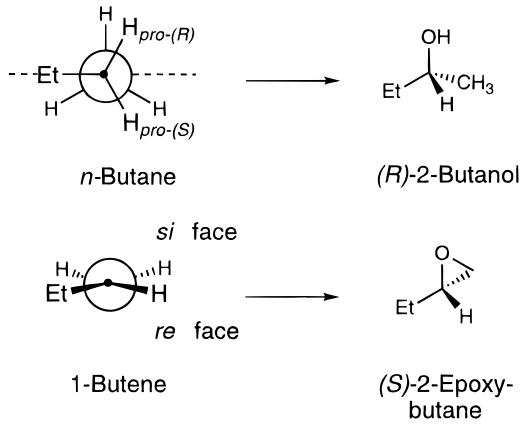


Figure 1. The equivalence of *n*-butane and 1-butene in the pMMO active site. Here, we consider oxidation of the C-2 position of each substrate, assuming retention of configuration in catalysis. Attack of the analogous face of substrates leads to an *(R)*-alcohol product for *n*-butane, or an *(S)*-epoxide product for 1-butene, as is observed.

The uncertainties in the reported ee values were determined by carrying out several replicates using a series of preparations of pMMO-containing membrane extracts. For example, the determination of the stereoselectivity of *n*-butane hydroxylation was repeated seven times with a total of three different pMMO preparations, and the ee value reported in Table 1 is an average of all the data accumulated. The standard deviation of data acquired from multiple experiments performed with samples from a given preparation was found to be small (typically this was found to be <2). As noted in Table 1, deviations between ee values acquired from different pMMO preparations were also acceptable. Other pMMO-mediated oxidations were carried out (at least) in duplicate, using different preparations, and again the variation between experiments was found to be small in comparison to the differences observed in the ee values for the different substrates. It should be noted that all experiments were carried out using membrane preparations of comparable activity. The use of membrane extracts of lower activity uniformly resulted in insufficient formation of products, which prohibited further stereochemical analysis.

The alkene substrates studied did not yield ee values greater than 40% and frequently yielded marginal stereoselectivity only. In part, the spectrum of ee values may be attributed to variable binding of the substrate (*vide infra*). In the case of propylene, variable binding modes may cause the near racemization observed if attack is conserved to a single side. If propylene binds either "methyl-first" or "vinyl-first" with nearly the same affinity, then the combination of binding modes would lead to both the (*R*)- and (*S*)-products. However, a preferred binding mode must exist, as completely racemic products are not attained. Further, the observed dominance of (*S*)-epoxide and (*R*)-alcohols suggests equivalent attack on the same face of butane and 1-butene, as shown in Figure 1. In the case of 1-butene, the binding mode (vinyl- or methyl-first) determines which product is formed and guides the stereospecificity. However, the binding of substrate is not rigorous. As demonstrated by the substrate 1,3-butadiene, the product 1,3-butadiene monoxide is produced with a small majority of the (*R*)-isomer. Such variable stereoselectivity has been observed previously in the epoxidation of a series of vinyl and allyl halide substrates oxidized by *M. trichosporium* (OB3b) in a whole-cell assay.[28,29]

(27) Parker, D. *J. Chem. Soc., Perkin Trans. 2* **1983**, 83.

(28) Ono, M.; Okura, I. *J. Mol. Catal.* **1990**, *61*, 113.

A Model for Dioxygen Activation and Alkane Hydroxylation by pMMO. As noted above, interpreting the observed regio- and stereoselectivities for a given substrate requires invoking substrate recognition and binding (or, how a substrate in presented within the active site) in addition to the intrinsic substrate reactivity (*i.e.*, mechanism). In the case of ethane, the stereochemical results offer a measure of the intrinsic reactivity because of the symmetry of the substrate. Thus, it appears that a concerted mechanism is at work for the oxygen atom insertion step, as previously proposed.[23] For other substrates, an understanding of the observed regioselectivity and stereoselectivity must include such considerations as the size and symmetry of the substrate molecule as well as the steric constraints imposed by the shape and symmetry of the active-site pocket.

We will first consider the steric requirements of the active site. As pMMO carries out regio- and stereoselective hydroxylation chemistry, it is evident that the active site must induce asymmetry upon a highly symmetric hydrocarbon substrate molecule. In addition to the simple steric considerations, which may be insufficient to limit rotational freedom of the pseudocylindrically symmetric hydrocarbon, the asymmetric catalysis may be facilitated by a copper−oxygen adduct which is of lower intrinsic symmetry. As mentioned above, pMMO is surmised to contain a trimeric copper cluster in the active site, and recent work has demonstrated the ability of dimeric[30−32] and trimeric copper clusters[33] to generate complexes that bind dioxygen and cleave the O−O bond by using electron equivalents from multiple copper atoms. In such model systems, the resulting adducts contain either a bis(μ_2-oxo) binuclear copper(III) core[30,32,34] or a bis(μ_3-oxo) trinuclear copper(II,II,III) core.[33] The displayed propensity of low-nuclearity copper clusters to form such bridged copper(III)−oxo adducts is helpful in considering the nature of pMMO-mediated oxygen activation and hydroxylation, particularly as copper is unlikely to form a terminal copper(III)−oxo species.[35] Further, within the regime of the $[Cu^{III}_2(\mu\text{-}O)_2]^{2+}$ core, Tolman and co-workers have noted the proclivity of the thermally unstable complexes to activate an internal C−H bond upon warming,[32,36,37] as have Stack and co-workers.[34] This process appears to have significant contributions from electrophilic oxo-centered acceptor orbitals.[31,37] Also, a single-electron reduction of this oxo-bridged moiety apparently further destabilizes the core, leading to unresolved decomposition of the dimeric complex prior to warming.[34] This new insight from model chemistry is useful in considering pMMO-mediated dioxygen activation and hydroxylation, although the model systems have a higher degree of symmetry than would account for the asymmetric catalysis described above, prompting consideration of an asymmetric dioxygen adduct for pMMO-mediated oxidations.

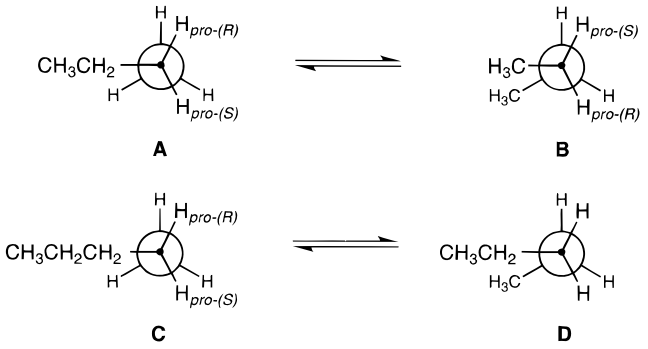


Figure 2. (Top) Here, we consider the possible hydroxylation of *n*-butane at either one of two carbon positions by presenting two translationally-related conformers of the substrate to the hydroxylating site. (Preferential hydroxylation at the C-2 position of the butanes is assumed.) Form **A** presents a *pro-(R)* hydrogen as depicted in Figure 1, while **B** presents a *pro-(S)* hydrogen at the same position within the pMMO active site. (Bottom) Using the same rationale for the oxidation of *n*-pentane, **C** presents a *pro-(R)* hydrogen, and **D** presents a C-3 hydrogen, which lacks prochirality.

Thus, in the presentation of a substrate molecule in the active site, we first consider a simple model for asymmetric hydroxylation that emphasizes attack on a single face of a hydrocarbon substrate (as in Figure 1) and attempt to rationalize the observed variation in ee value due to variability in the substrate binding. In the case of propylene, either the *re* or the *si* faces may be presented in essentially the same orientations, and this should lead to a racemic mixture of the *(R)*- and *(S)*- epoxides, as observed. Similarly, the formation of 3-butan-2-ol and 1-epoxybutane in essentially equal proportions can be understood in terms of such a binding scheme and equilibrium. If the asymmetric hydroxylation or epoxidation involves attack on the same face of the hydrocarbon substrate as in Figure 1, the stereochemical configuration of the alcohol and the epoxide should be *(R)* and *(S)*, respectively, as observed, if the attack proceeds with retention of configuration.

A possible scheme to account for the stereoselectivity of butane hydroxylation is shown in Figure 2. As shown therein, butane can be thought of as having two binding modes, **A** and **B**, which differ *translationally* as the butane molecule slides forward and backward in the binding site, such that either a *pro-(R)* (**A**) or a *pro-(S)* hydrogen (**B**) is presented in a similar orientation within the active site. Presuming catalysis proceeds with retention of configuration, as was demonstrated for ethane,[23] **A** leads entirely to *(R)*-butan-2-ol and **B** leads to *(S)*-butan-2-ol. Thus, the observed stereoselectivity may be rationalized if a butane molecule shifts within the equilibrium by a small translational motion. However, such a binding scheme fails to account for the hydroxylation of pentane, which would similarly bind in forms **C** and **D**, yielding *(R)*-pentan-2-ol and pentan-3-ol. This model predicts the formation of pentan-3-ol in analogous fashion to the formation of *(S)*-butan-2-ol. Based on the observed ee value for butane hydroxylation, pentan-3-ol should constitute 27% of the hydroxylation products. Yet no pentan-3-ol is observed. Similarly, propane should reorient in the binding site, and the equilibrium would yield ∼27% of the total products as propan-1-ol; yet, propan-1-ol has been detected at trace levels only. Thus, this "translational binding model" cannot account for the regiospecificity observed as long as the substrates are assumed to freely reorganize within the active site.

While it seems unlikely that pentane would be able to reorganize due to steric reasons (as compared to butane), propane should be allowed to move quite freely. However, the stringent oxidation of propane at the C-2 position can be

(29) Seki, Y.; Shimoda, M.; Sugimori, D.; Okura, I. *J. Mol. Catal.* **1994**, *87*, L17.
(30) Halfen, J. A.; Mahapatra, S.; Wilkinson, E. C.; Kaderli, S.; Young, V. G.; Que, L.; Zuberbühler, A. D.; Tolman, W. B. *Science* **1996**, *271*, 1397.
(31) Mahapatra, S.; Halfen, J. A.; Wilkinson, E. C.; Pan, G.; Wang, X.; Young, V. G.; Cramer, C. J.; Que, L.; Tolman, W. B. *J. Am. Chem. Soc.* **1996**, *118*, 11555.
(32) Mahapatra, S.; Young, V. G.; Kaderli, S.; Zuberbühler, A. D.; Tolman, W. B. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 130.
(33) Cole, A. P.; Root, D. E.; Mukherjee, P.; Solomon, E. I.; Stack, T. D. P. *Science* **1996**, *273*, 1848.
(34) Stack, T. D. P. Personal communication.
(35) Mayer, J. M. *Comments Inorg. Chem.* **1989**, *8*, 125.
(36) Mahapatra, S.; Halfen, J. A.; Wilkinson, E. C.; Que, L.; Tolman, W. B. *J. Am. Chem. Soc.* **1994**, *116*, 9785.
(37) Mahapatra, S.; Halfen, J. A.; Tolman, W. B. *J. Am. Chem. Soc* **1996**, *118*, 11575.

explained by invoking some feature of the protein environment, such as a small hydrophobic pocket that can accommodate a methyl group of propane, but not any group larger. In such a scenario, this small pocket would hold a propane molecule in a position that exposes the C-2 position for alkane substrates and preferentially deters reorganization of the substrate. Thus, attack at the C-2 position is enforced. However, when the substrate is not fully extended into this pocket, some reactivity can occur at the C-1 position, as is observed in Table 1. With these types of boundary conditions, it seems that most substrates are bound in a single translational conformer preferentially, with some potential for translation reorganization that results in the observed regiospecificity. The regioselectivity observed would then reflect the free energy difference between the various modes of presenting the hydrocarbon substrate to the hydroxylation site, as imposed by the steric constraints.

Further consideration of the substrate range displayed by pMMO can aid in revising the model proposed in Figure 2. Linear straight-chain hydrocarbons no more than five carbons in length appear to be favored substrates. As the data seem to preclude the notion of significant translational motion of the substrate within the binding site, it may be concluded that the active-site pocket in pMMO has a limited depth and is closed at one end in the small pocket mentioned above, such that longer hydrocarbon chains (*i.e.*, hexane, heptane, etc.) are not appropriate substrates because their size interferes with a conformational change of the binding pocket that is required for catalysis. This suggestion is supported by the propensity for small 1-alkynes, such as acetylene[38] and 1-butyne, to act as suicide substrates, while the larger putative suicide substrate 1-hexyne does not interfere with normal catalysis.[39] These observations further confirm that pMMO contains a small active site that binds substrate very tightly, allowing only minimal translational reorganization of substrate molecules.

With these considerations in mind, Figure 3 presents a further refinement of a binding scenario in which either (*R*)- or (*S*)-alcohol product results *via* a nonspecific oxidation of substrate molecules bound with the same translational orientation. Within this model, subtle differences in binding mode influence the stereoselectivity of the reaction and, potentially, the mechanism itself. Figure 3 implicitly assumes that the translational freedom allowed in Figure 2 is minimized, but, as before, substrates interact with a single face of a dicopper core. Further, we propose that steric interactions, and the conformation constraints of the protein environment, allow for two binding modes (**I** and **II**) to position either the *pro-(R)* or *pro-(S)* hydrogen such that attack by the dicopper core proceeds, but the overall preference in substrate binding (**I**) yields the observed preferential oxidation at the *pro-(R)* hydrogen. The two binding modes also correspond to two active-site conformations that differ slightly, yielding the overall preference in exposing the *pro-(R)* or *pro-(S)* hydrogen while maintaining the hydrophobic contacts that result in preferential attack at the C-2 position. The two modes clearly will be close in energy, to be consistent with the ee ratios reported in Table 1. Even in the case of the greatest stereoselectivity, an ee of 80% for *n*-pentane hydroxylation, the 1:9 distribution of (*S*)- to (*R*)-alcohol can be accounted for by a small free energy differences between the substrate conformers: a mere 5.75 kJ/mol, which is less than 2 kT per molecule at 315 K. The analogy between alkane and alkene binding modes illustrated in Figure 1 still holds within this scheme, although it can be imagined that, if pMMO does bind alkanes very tightly, the increased rigidity of the olefin may reduce the ability of the substrate to conform to the preferred binding modes allowed by the conformational flexibility of the active site. Thus, the stereoselectivity of alkene epoxidation would be reduced, as is observed.

The Dioxygen Adducts and Mechanistic Hypotheses. Finally, we turn to the question of the dioxygen adduct that may be involved in catalysis. The observed thermal decomposition of model complexes containing a $[Cu^{III}_2(\mu\text{-}O)_2]^{2+}$ core is satisfied by the trinuclear copper cluster implicated as the active site here. In particular, the findings of Stack and co-workers regarding bolstered reactivity of $[Cu^{III}_2(\mu\text{-}O)_2]^{2+}$ upon a one-electron reduction suggest that injection of an additional electron into the reaction site, potentially by another copper ion, may enhance (or, indeed, be required for) reactivity. Such a process would imply that the redox states of the two copper ions within the core need not be identical in conventional formalisms, *i.e.*, in Figure 3, $m \neq n$. In addition, proximity and orientation of the substrate can guide the decomposition pathway, as the binding of substrate and its potential for slight reorganization, can affect both the character of the resulting dicopper core and which oxygen atom of the dicopper core is incorporated into product, thereby leading to *(R)*- and *(S)*-isomers. The correlation between oxidation state, substrate orientation, and reactivity is specifically depicted in Scheme 1.

This catalytic hypothesis is attractive in that it directly links binding of a small substrate to reactivity: substrate binding can be thought of as triggering the redox linkage necessary for the further reduction of the $[Cu^{III}_2(\mu\text{-}O)_2]^{2+}$ core, thereby increasing the energy of the ground state electronic structure of the intermediate, lowering the kinetic barrier for catalysis.

Generally speaking, this binding model can serve as suitable grounds for subsequent mechanistic hypotheses. For example, conformer **I** may be better suited for a concerted reaction process, as it agrees with the steric constraints of the preferred transition state structure proposed previously[40] for the insertion of an electrophilic oxygen atom into a methylene C−H bond of *n*-butane by a solvated oxenoid species. Interestingly, this type of insertion process has already been proposed for sMMO by Shestakov and Shilov.[41] While Tolman and co-workers have postulated that the bridging oxygen atoms have significant electrophilic character,[31,37] we cannot rigorously discount other possibilities, such as the invocation of the direct formation of a high-valent organocopper intermediate, as suggested by Barton and co-workers.[42,43] The direct involvement of one or both of the proposed oxo bridges is also circumspect, as a concerted process may be accommodated by either possibility: one satisfying mechanism couples nucleophilic attack of the substrate *pro-(R)* proton by one bridging oxygen atom to the concomitant electrophilic attack of the methylene carbon by the other bridging oxygen, thus requiring a cyclic transition state (central pathway). However, direct oxygen insertion may involve only a single oxygen atom (left pathway). A radical rebound mechanism may account for the findings as well, as long as the active site poses severe steric constraints on the structure of resulting radical intermediates and/or the rebound rate for methane and ethane hydroxylation is too large to allow the extended existence of a discreet population of $R^{\bullet}$ species (right pathway). However, the previous study of chiral ethane oxidation, described earlier,[23] clearly demonstrates that the

(38) Prior, S. D.; Dalton, H. *FEMS Microbiol. Lett.* **1985**, *29*, 105.
(39) Data not shown.
(40) Bach, R. D.; Andrés, J. L.; Su, M.-D.; McDouall, J. J. W. *J. Am. Chem. Soc.* **1993**, *115*, 5768.
(41) Shestakov, A. F.; Shilov, A. E. *J. Mol. Catal. A* **1996**, *105*, 1.
(42) Barton, D. H. R.; Bévière, S. D.; Chavasiri, W.; Csuhai, É.; Doller, D. *Tetrahedron* **1992**, *48*, 2895.
(43) Barton, D. H. R.; Csuhai, E.; Doller, D. *Tetrahedron Lett.* **1992**, *33*, 4389.

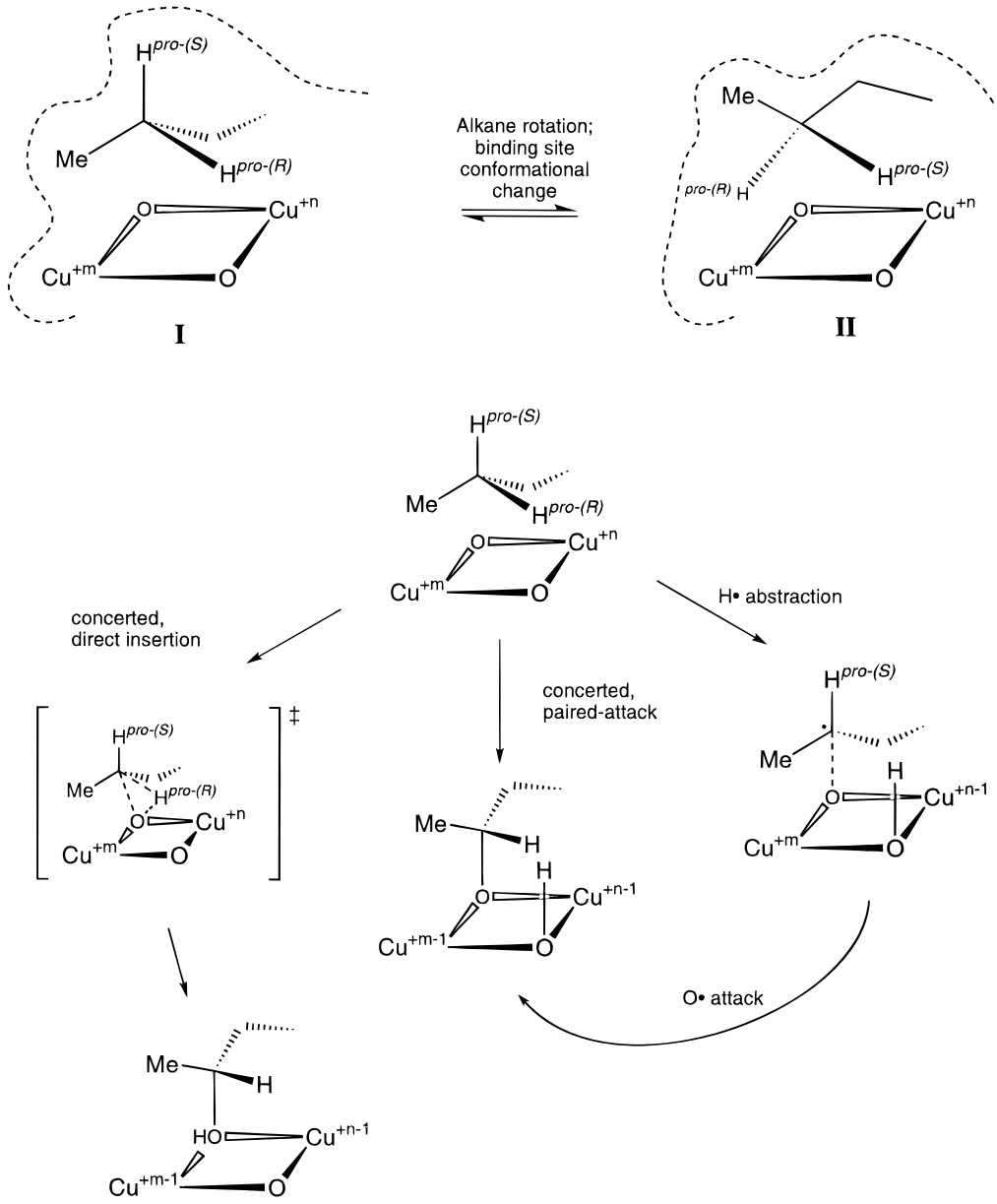
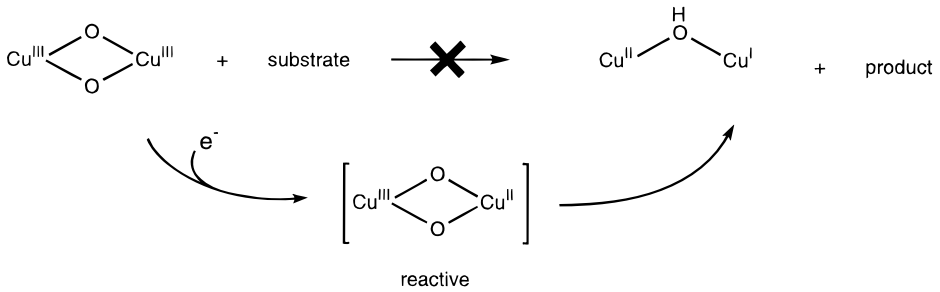


Figure 3. (Top) An alternative substrate binding scheme that does not allow for translational motion between conformers. Forms **I** and **II** are related by differential insertion into the active site, which alternatively places either the *pro-(R)* or the *pro-(S)* hydrogen of the C-2 position in close proximity to an oxygen atom of a proposed dicopper−oxo core, as found in model complexes previously. (Bottom) Mechanistic hypotheses based on the above binding model. This model readily allows for concerted mechanisms, such as a direct insertion of an activated oxygen atom into the *pro-(R)* C−H bond (left pathway) or a concerted pairwise process (middle pathway). A nonsynchronous mechanism such as hydrogen atom abstraction followed by attack of an oxygen-based radical (right pathway) is equally feasible.

Scheme 1



lifetime of such an alkyl radical would have to be shorter than $<1 \times 10^{-14}$ s, implying that the contribution of such a process is most unlikely in the oxidation of ethane.

Thus, the above discussion describes how the competition between substrate binding modes may affect the observed stereoselectivity, and how a number of possibilities might be consistent with a hydroxylation mechanism that proceeds through retention of configuration, as has been ascribed to the hydroxylation of ethane.[23] However, as just discussed, the regio- and stereochemical characterization of reaction products cannot rigorously exclude one mechanism or another, although a model describing an appropriate binding mode can be developed. It

can be noted that, if a substrate is unable to adopt the preferred conformer for a given mechanism, it is quite possible that the hydroxylation chemistry proceeds through one of the alternative processes. Indeed, the existence of parallel pathways cannot be ruled out at this time, and it seems most probable that the electronic structure of the activated oxygen species will be controlled by the arrangement of the inner-sphere ligands around the copper atoms. This means that the steric effects for a given substrate molecule may perturb the electronic structure of the site and, thereby, perturb the reactivity of the site as well.

Conclusions

The observed regio- and stereospecificity of alkane hydroxylation and alkene epoxidation mediated by pMMO are reported here. This study has delineated the issues of substrate binding in the activation of alkanes by pMMO, generating a model for substrate binding that can be related to known copper−dioxygen chemistry and the previously proposed mechanism for alkane activation. It is apparent that multiple pathways for alkane hydroxylation and alkene epoxidation contribute to the observed regio- and stereoselectivity and that these results may vary between substrates in a manner that is determined by the substrate binding mode. Specifically, while pMMO demonstrates stringent substrate specificity, we can rationalize a preferential binding model that presents a substrate such that nonspecific, concerted catalysis yields stereoselectivities that vary with substrate size and shape. Moreover, in the case of alkane hydroxylation, the binding mode of the substrate at the active site can expose either the *pro*-(*R*) or *pro*-(*S*) C−H bond at the C-2 position in a substrate-specific manner. Although ethane oxidation appears to proceed through a concerted mechanism, whether this is rigorously the case for other alkanes remains to be seen. Further studies using diagnostic kinetic isotope techniques will, hopefully, elucidate the mechanistic aspects of this problem, expanding on the model presented herein.

Acknowledgment. This work was supported by the National Institutes of Health (GM 22432 to S.I.C.). L.T. acknowledges support from the California Museum of Science and Industry Foundation. H.-H.T.N. is the recipient of a National Research Service Predoctoral Award and a W.R. Grace Fellowship. S.J.E. and S.I.C. would like to acknowledge several helpful discussions with Professor T.P.D. Stack (Stanford University) and Professor William B. Tolman (University of Minnesota). Finally, we are indebted to Dr. Peter Green and the Bank of America Environmental Analysis Center (Caltech) for technical assistance.

JA971049G