# Probing the Mechanism of C−H Activation: Oxidation of Methylcubane by Soluble Methane Monooxygenase from *Methylosinus trichosporium* OB3b[†]

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ABSTRACT: The soluble form of methane monooxygenase (MMO) isolated from methanotrophic bacteria catalyzes the $O_2$-dependent conversion of methane to methanol, as well as the adventitious oxidation of many other hydrocarbons. In past studies, it was reported that the oxidation reaction of methylcubane, a radical clock substrate, catalyzed by MMO from *Methylococcus capsulatus* (Bath) gave only cubylmethanol as the product rather than methylcubanol(s) or rearranged products characteristic of a radical formed on the methyl group [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]. Such a substrate radical intermediate would be expected if the mechanism of MMO involves hydrogen atom abstraction as indicated by many previous mechanistic studies. Here it is shown that the reaction of methylcubane with the reconstituted MMO system from *Methylosinus trichosporium* OB3b yields both cubylmethanol and methylcubanols, with methyl hydroxylation favored over cubyl hydroxylation. This unexpected regioselectivity indicates steric effects on the reaction in agreement with past product distribution studies. In addition, the apparent majority product of the reaction is tentatively assigned as one of the possible rearranged products for this radical probe, on the basis of gas chromatography and mass spectrometry data. This result suggests the formation of a radical intermediate in the reaction, thus supporting a radical-based mechanism for this form of MMO.

The soluble MMO[1] system found in methanotrophic bacteria efficiently catalyzes the oxidation of methane to methanol in initiating a pathway that provides carbon and energy for the organism (*1*, *2*).

$$NADH + CH_4 + O_2 + H^+ \xrightarrow{MMO} NAD^+ + CH_3OH + H_2O$$

This extraordinary ability of MMO to catalyze the fissure of an unactivated C−H bond of methane extends to a wide spectrum of other hydrocarbons that serve as adventitious substrates (*3*−*6*), a property which has been widely used to probe the molecular chemistry of C−H oxidation (*3*, *4*, *6*−*14*). In recent years, extensive structural and mechanistic studies have been conducted with the soluble MMOs isolated from *Methylosinus trichosporium* OB3b (*7*, *15*) and *Methylococcus capsulatus* (Bath) (*1*, *8*). For both organisms, the soluble MMO system was found to consist of three protein components: a hydroxylase termed MMOH, an NADH-coupled reductase, and a regulatory component termed MMOB. MMOH contains a bis-$\mu$-hydroxo-bridged diiron cluster at the active site which is responsible for oxygen activation and substrate oxidation (*16*, *17*). X-ray crystallography studies of MMOHs isolated from *M. capsulatus* (*18*, *19*) and *Me. trichosporium* (*20*) show that despite the fact that they originate from widely divergent organisms, the enzymes have nearly identical structures.

Kinetic and spectroscopic studies of alkane hydroxylation by MMO have indicated that the reaction proceeds through the following major processes (Figure 1) (*21*−*25*): starting with the enzyme in its diferric resting state ($\mathbf{H_{ox}}$), (i) reduction of the MMOH diiron cluster to the diferrous state ($\mathbf{H_{red}}$), (ii) reaction with $O_2$ to form successive intermediates **O**, **P***, and **P**, (iii) breaking of the O−O bond to form intermediate **Q**, (iv) reaction with alkane substrate to yield the alcohol product−enzyme complex termed intermediate **T**, and, finally, (v) release of product and regeneration of the resting state enzyme.

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[1] Abbreviations: MMO, soluble methane monooxygenase; P450, cytochrome P450 monooxygenase; MMOH, hydroxylase component of methane monooxygenase; MMOB, B component of methane monooxygenase; $\mathbf{H_{ox}}$, diferric state of MMOH; $\mathbf{H_{red}}$, diferrous state of MMOH; **O**, **P***, **P**, **Q**, **R**, and **T**, intermediates of the MMO catalytic cycle termed compounds O, P*, P, Q, R, and T, respectively; EXAFS, extended X-ray absorption fine structure; KIE, kinetic isotope effect; MOPS, 3-(*N*-morpholino)propanesulfonic acid; GC−MS, gas chromatography−mass spectrometry; $k_r$, intrinsic rate of rearrangement for a radical clock substrate; **RP1**, **RP2**, and **RP3**, possible hydroxylation products of methylcubane resulting from the rearrangements of a cubylcarbinyl radical and cation; *m*/*z*, mass/charge ratio; relative intensity, percentage intensity of a fragment ion in a mass spectrum relative to the most abundant fragment ion.

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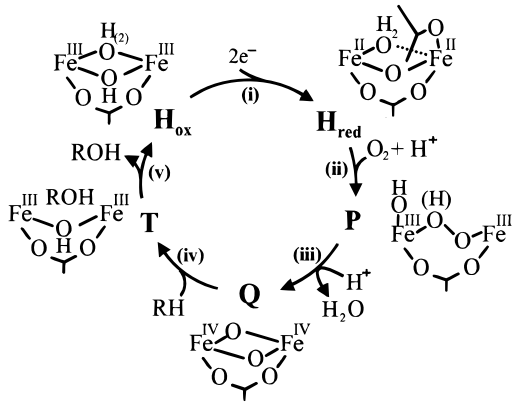


FIGURE 1: MMO catalytic cycle. MMOH is represented by the active site diiron center. The structures of the cycle reactants that are shown reflect our current knowledge based on crystallographic, spectroscopic, and kinetic studies. For simplicity, not all the intermediates that were observed are included and not all iron ligands are shown.

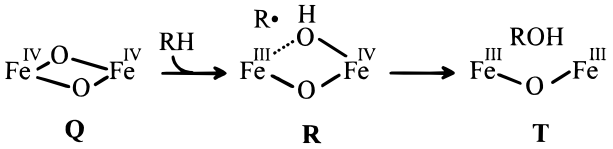


FIGURE 2: Conventional oxygen rebound mechanism for the C−H oxidation step of the MMO reaction. The reaction is postulated to proceed through hydrogen atom abstraction to form a substrate radical and intermediate **R**, the radical complex. The recombination of the substrate radical with the hydroxyl group in **R** yields **T**, the terminal product complex.

Intermediates **P** and **Q** can be formed and trapped in high yield in transient kinetic studies, allowing their structures to be evaluated spectroscopically (*21*, *26*). The Mössbauer parameters of **P** show that it is likely to contain a peroxy or hydroperoxy adduct of a binuclear ferric cluster (*26*, *27*). Mössbauer and EXAFS spectra of **Q** show that it is a unique species containing two Fe(IV)s bridged by at least two single oxygen atoms (*22*, *27*). As such, it is electronically equivalent to the oxidizing species in the cytochrome P450 systems thought to be an oxo−Fe(IV)−$\pi$ cation radical (*28*). Accordingly, **Q** appears to be the species that reacts directly with hydrocarbons. This view is supported by the observations that the rate of its decay reaction is linearly dependent on the concentration of substrate present and that the active site-bound product (in **T**) is formed at the same rate as **Q** decays (*21*). Unfortunately, the chemical steps that occur during the conversion of **Q** to **T** occur too rapidly to be observed directly. We have proposed that the nature of these steps has a molecular mechanism similar to that of P450, as indicated by the overall electronic similarity of the reactive species for P450 and MMO (*21*, *27*−*29*). In the case of P450, the hydrocarbon oxidation process is thought to involve hydrogen atom abstraction from the hydrocarbon substrate by the oxo−Fe(IV)−$\pi$ cation radical species, yielding an intermediate substrate radical. Rebound of the hydroxyl group from the iron to the substrate radical would complete the reaction (*30*). The equivalent hydrogen abstraction process in the MMO cycle (Figure 2) is evidenced by the observation of an exceptionally large deuterium KIE during **Q** decay in the presence of deuterated methane (*31*) which suggests the occurrence of a discrete C−H bond breaking step. In addition, the formation of a substrate radical (Figure 2), the product of the initial step, is supported by the observation of the partial inversion of stereochemistry in the MMO-catalyzed oxidation of chiral 1-[$^1$H,$^2$H,$^3$H]ethane (*9*).

Another approach to the evaluation of substrate radical intermediates in MMO catalysis has been the use of "radical clock" substrates. These substrates undergo characteristic reorganization reactions when a radical is formed in their structure. Since the reorganization occurs at a distinct rate, both the existence and the lifetime of a radical intermediate can be determined (*32*). Unfortunately, the results with various clock oxidation experiments for MMO do not support the same mechanism. An early report showed that the oxidation of 1,1-dimethylcyclopropane catalyzed by *Me. trichosporium* MMO gave rise to rearranged products indicative of the formation of substrate radical and cationic intermediates during reaction (*10*). In contrast, more recent studies with radical clock substrates capable of detecting even shorter-lived radicals, such as aryl-substituted methylcyclopropanes, failed to evoke any evidence of a radical intermediate during oxidation catalyzed by *M. capsulatus* MMO (*11*). Oxidation of one aryl-substituted methylcyclopropane catalyzed by *Me. trichosporium* MMO gave a small amount of rearranged product, but the lifetime of the radical intermediate was predicted to be too short for the rebound mechanism described above. One potential difficulty with aryl-substituted radical clocks is that they may be distorted in the enzyme active site, causing slower reorganization than expected. This has been addressed by using methylcubane as a radical clock. The rigid structure of this molecule prevents distortion, but a radical formed on the methyl group has the potential to initiate opening of the cubane structure. Again, no rearrangement was observed for the reaction catalyzed by the enzyme isolated from *M. capsulatus* (*14*).

Because the results of these radical clock studies appear to be very sensitive to both the substrate and the enzyme that are used, we have investigated the hydroxylation reaction of methylcubane catalyzed by MMO from *Me. trichosporium* OB3b. The results differ significantly from those reported for *M. capsulatus* MMO despite the similar structures of the two enzymes and the occurrence of similar reaction cycle intermediates. In light of previous studies, the current results support a model in which subtle differences in the accessibility to the reactive species in the active site mask inherently similar, radical-based chemical mechanisms of MMO.

## MATERIALS AND METHODS

*Enzyme Preparation*. The components of MMO were purified to homogeneity from *Me. trichosporium* OB3b. Details of the growth of the bacterial strains and protein purification procedures have been published previously (*33*, *34*). The specific activities of MMOH preparations used for the experiments were in the range of 600−1000 nmol min$^{-1}$ mg$^{-1}$ when they were determined at 23 °C using furan as the substrate as described previously (*34*).

*Chemicals*. Methylcubane and cubylmethanol were generously provided by M. Newcomb (Wayne State University, Detroit, MI). MOPS was purchased from Sigma (St. Louis, MO), and all other chemicals were from Aldrich (Milwaukee, WI) and were used without further purification. Water was deionized and glass-distilled.

*Enzymatic Oxidation.* A typical experiment was conducted in the following manner. Due to the low solubility of methylcubane, a stock substrate-saturated solution was made prior to use. Approximately 3 mg of substrate was added to 2 mL of oxygen-saturated 50 mM MOPS buffer (pH 7.5) in a Teflon-sealed container, in which the mixture was then stirred for 12 h. The enzyme-catalyzed reaction was conducted in Teflon-sealed reaction vials containing 6 mg of MMOH, 0.7 mg of MMOB, and 1.5 mg of reductase in 0.5 mL of the substrate buffer. It was necessary to preincubate the enzyme in the substrate solution on ice for 15 min to allow equilibration of the reactants. The reaction vial was then shaken at 30 °C in a constant-temperature bath, and the reaction was initiated by the addition of 5 $\mu$L of 500 mM ethanol-free NADH to the vial. To check the stability of cubylmethanol under experimental conditions, the enzyme-catalyzed reaction of cubylmethanol was conducted in a similar manner.

*Product Analysis.* To obtain the time course for the formation of products, the reaction was quenched at appropriate time points by mixing 0.1 mL of reaction aliquot with 0.1 mL of chloroform that contained a small amount of cyclooctane as an internal standard for GC analysis. The mixture was vortexed and then centrifuged for 1−2 min, and then 1−2 $\mu$L of the organic extract was injected into the gas chromatograph without further treatment. An aliquot was also taken before the addition of NADH and treated similarly to obtain zero time data point. GC analysis was conducted on a Hewlett-Packard model 5890 gas chromatograph equipped with an FID detector and a capillary DB-1 column. Relative yields of product were determined by integration of GC peak areas.

For the GC−MS assay, reaction mixtures were extracted twice with equal volumes of chloroform. The organic extracts were combined and concentrated slowly under argon, and then subjected to GC−MS analysis. GC−MS was performed using a Carlo-Erba gas chromatograph with a capillary DB-5 column interfaced with a Kratos MS-25 spectrometer (EI, 70 eV).

## RESULTS

Methylcubane potentially can be hydroxylated at two types of positions, the C−H of the side chain methyl group and the C−H of the cubyl group, giving cubylmethanol and different methylcubanols as products, respectively (Figure 3A). If the hydroxylation reaction involves hydrogen atom abstraction, substrate radical intermediates would be formed. Although the cubyl radical retains its integrity in solution, the cubylcarbinyl radical is known to rearrange rapidly, as shown in Figure 3B, at a rate of $2.8 \times 10^{10}$ s$^{-1}$ at 30 °C (*35*, *36*). Consequently, products such as **RP1** and **RP2** are expected if radicals live long enough to rearrange. If cationic intermediates are formed during reaction, production of **RP3** would be possible, since the cubylcarbinyl cation is also known to rearrange (Figure 3C) (*35*).

Upon reaction with the reconstituted MMO from *Me. trichosporium* OB3b, NADH, and $O_2$, methylcubane afforded several products as illustrated in the reaction GC trace and the product profiles (Figure 4A,B). GC−MS analysis revealed that the species in GC elution peaks **1**−**3** exhibited parent ions at *m*/*z* 134 ($C_9H_{10}O$) (Figure 4C), consistent with

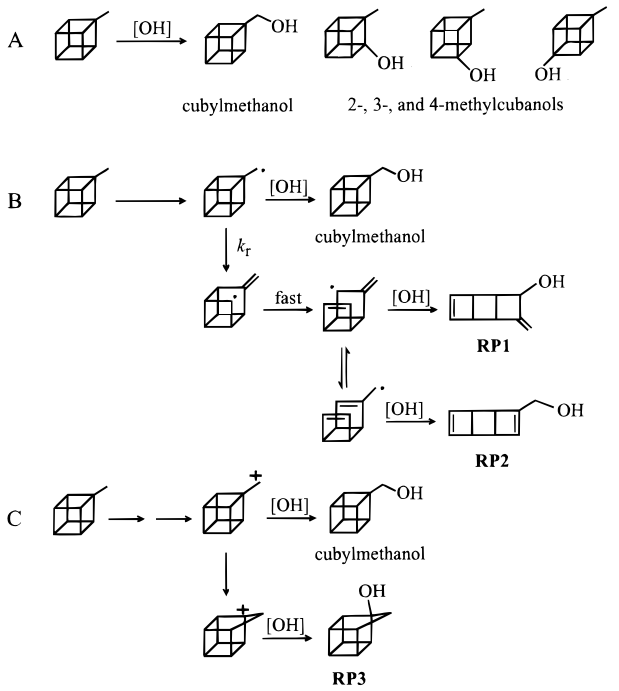


FIGURE 3: Potential products from (A) direct hydroxylation of methylcubane, (B) the formation of a cubylcarbinyl radical intermediate that rearranges rapidly, and (C) the formation of a cubylcarbinyl cation intermediate that can also rearrange.

them being hydroxylation products of the substrate ($C_9H_{10}$). The small peaks between **1** and **2** are postulated to be products from secondary hydroxylation because they give parent ions at *m*/*z* 150 ($C_9H_{10}O_2$) in their mass spectra (data not shown).

Production of cubylmethanol is confirmed by comparing GC retention times and MS properties of product **2** with those of the authentic standard. Its mass spectral fragmentation pattern also appeared to be the same as that of cubylmethanol acetate (Table 1), which was analyzed as a derivatized product in P450- and *M. capsulatus* MMO-catalyzed methylcubane oxidations (*14*).

At approximately the retention time corresponding to the single peak of product **3** in the GC trace from a DB-1 column, three overlapping peaks were detected from a DB-5 column during GC−MS analysis (shown in the inset of panel Figure 4A). Mass spectral data revealed that the species in each peak had the same fragmentation ions, including the parent ions at *m*/*z* 134, but with slightly different relative intensities (Table 1). We believed that these species are the three possible methylcubanols resulting from hydroxylation at cubyl C−H positions, which were also produced in P450-catalyzed methylcubane oxidations (*14*). Because the three putative cubyl alcohols were poorly resolved and had very similar mass spectra, it was impossible to differentiate between them without authentic standards. Our assignment of products in **3** to cubyl alcohols was further supported by the agreement of their mass spectra with those of methylcubanol acetates observed in the P450 reaction (Table 1), specifically the same overall fragmentation pattern (combination of *m*/*z* 91, 105, and 119) and the highly characteristic $(M - 15)^+$ peak (*m*/*z* 119) resulting from the loss of the methyl group. Interestingly, production of methylcubanols was not detected in the reaction of methylcubane with MMO from *M. capsulatus* (*14*).

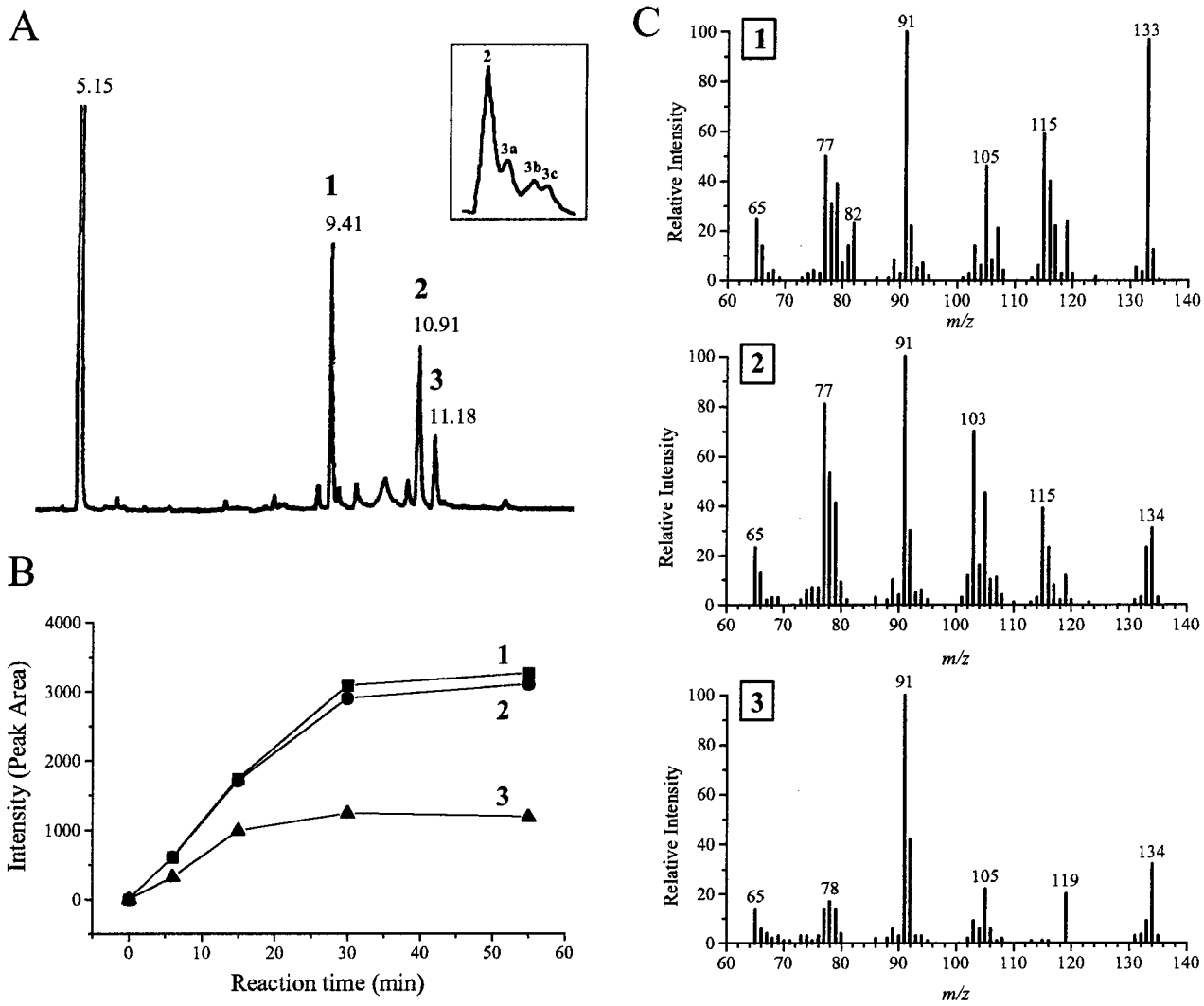


FIGURE 4: (A) Typical gas chromatogram from a DB-1 column of the chloroform extract of methylcubane hydroxylation catalyzed by MMO from *Me. trichosporium* OB3b. The peaks with retention times of 9.41 (product **1**), 10.91 (product **2**), and 11.18 min (product **3**) and the small peaks between **1** and **2** were not present in the control experiment carried out under the same conditions except in the absence of NADH (zero time data point). The peak at 5.15 min is due to unreacted methylcubane. The inset shows the three peaks detected in place of product **3** from mass spectrometer analysis following separation by a DB-5 GC column and detection as total ion count from the MS (see Materials and Methods). (B) Time course for the formation of **1**−**3** during the reaction. (C) Mass spectra of species **1**, **2**, and **3a**, one of three composite species of **3**.

Product **1** has GC elution properties quite different from those of **2** and **3**, suggesting that it is structurally distinct. Nevertheless, it exhibits the same parent ion at *m*/*z* 134, showing that it is also a hydroxylation product of the substrate. As indicated in the product profile (Figure 4B), product **1** appeared to be formed at the same rate as product **2**, cubylmethanol, whereas the hydroxylation at cubyl positions (to form **3**) occurred at a much slower rate, assuming the products have similar GC response factors. In a control experiment, the oxidation of cubylmethanol by MMO apparently gave a double-hydroxylation product(s) with a longer retention time (data not shown). This reaction is very slow, possibly for the same reason that methylcubanols were slowly formed. The fact that this control did not give a product eluting at the position of peak **1** indicated that the origin of the species in peak **1** is neither enzymatic nor nonenzymatic isomerization of product **2**, but rather hydroxylation of methylcubane. Given that the species in peak **1** is neither of the direct hydroxylation products, it seems likely that **1** is a rearranged product for this radical probe. This is supported by the nearly identical formation rates of **1** and **2**, consistent with them originating from the same intermediate. This is the expected result if the common intermediate is the cubylcarbinyl radical or cation.

Among the three possible rearrangement candidates, product **1** was tentatively assigned as **RP2** on the basis of several considerations. First, as shown in the reaction GC trace (Figure 4A), products **2** (cubylmethanol) and **3** (methylcubanols) have similar GC retention times (10.91 and 11.18 min, respectively, from the DB-1 column), possibly due to the fact they share the basic cubane structure. In contrast, product **1** elutes from the column much earlier (9.41 min) and was separated from its isomers, **2** and **3**, by likely secondary hydroxylation products of **1**.[2] This chromatographic behavior is indicative of a structure (shape and size)

[2] These products with retention times longer than that of **1** may arise from further hydroxylation of **1** just as in a control experiment enzyme-catalyzed oxidation of cubylmethanol apparently gave double hydroxylation product(s) with a retention time longer than those of **2** and **3**. The double-hydroxylation product(s) of **2** was not observed in the enzyme-catalyzed oxidation of methylcubane, probably due to the low concentration of **2** and its slow turnover rate.

Table 1: Comparison of Mass Spectral Data

| |
|---|
| cubylmethanol and **2**[a] |
| *m*/*z* (relative intensity) |
| 34 (30), 115 (39), 103 (70), 91 (100), 77 (81), 65 (23) |
| cubylmethanol acetate[b] |
| *m*/*z* (relative intensity) |
| 133 (9), 115 (100), 103 (16), 91 (18), 77 (18), 65 (4), 51 (8) |
| **3** (methylcubanols)[c] |
| *m*/*z* (relative intensity) |
| 134 (32), 119 (20), 105 (22), 91 (100), 78 (18), 65 (14); |
| 134 (26), 119 (10), 105 (46), 91 (100), 77 (32), 65 (19); |
| 134 (39), 119 (30), 105 (45), 91 (100), 77 (28), 65 (21) |
| methylcubanol acetates[d] |
| *m*/*z* (relative intensity) |
| 133 (65), 119 (74), 105 (80), 94 (36), 91 (100), 77 (80), 51 (30); |
| 133 (60), 119 (83), 105 (73), 94 (43), 91 (100), 77 (65), 51 (23); |
| 133 (56), 119 (76), 105 (92), 94 (83), 91 (100), 77 (87), 65 (28), 51 (31) |

[a] MS data of the authentic cubylmethanol and product **2** of methylcubane oxidation catalyzed by *Me. trichosporium* MMO. The two mass spectra were indistinguishable. [b] MS data of the derivatized methyl hydroxylation product of methylcubane oxidation catalyzed by P450s and *M. capsulatus* MMO (*14*). [c] MS data of products **3** of methylcubane oxidation catalyzed by *Me. trichosporium* MMO, which were assigned as methylcubanols. [d] MS data of derivatized cubyl hydroxylation products of methylcubane oxidation catalyzed by P450s (*14*).

that is different from a cubane unit. The distinctive tricyclic structures of **RP1** and **RP2** are consistent with the observed chromatographic behavior.

Another rationale for assignment of **1** as **RP2** is derived from an examination of the mass fragmentation patterns. Because of the highly cyclic nature of all the compounds examined here, their mass spectra give similar fragments. However, some distinctive mass fragment peaks provide information about structure. The mass spectrum of product **1** has an extremely intense $(M - 1)^+$ peak (*m*/*z* 133, relative intensity of 97), whereas those of the other products do not (Figure 4C). The $(M - 1)^+$ ion can usually be seen in the mass spectra of primary and secondary alcohols, but it is generally not very intense. The unsaturated structures in **RP1** and **RP2** give them potential to have a prominent $(M - 1)^+$ fragment by losing H to form a conjugated C═C−C═O system, which would stabilize the fragment ion, and thus increase the probability of its appearance. On the other hand, for a tertiary alcohol, the $(M - 1)^+$ peak is usually small or undetectable, as in the case of products **3** which have very small $(M - 1)^+$ peaks in agreement with their assignments as cubyl alcohols. One would expect **RP3** to have an $(M - 1)^+$ peak with an intensity similar to those of **3**, again making it unlikely to be product **1**.

A third rationale is based on the masses of the mass fragments that are observed. As observed for cubylmethanol, product **1** gives a prominent *m*/*z* 115 fragment. This is consistent with the loss of terminal water, suggesting that product **1** is probably a primary alcohol, and thus more likely to be **RP2**. This fragment is not observed from products **3** (methylcubanols), because they are tertiary alcohols. Furthermore, one would expect to observe predominant fragments due to the loss of the side chain for **RP1**, just as the intense *m*/*z* 119 peak was observed for **3**.

In summary, **RP2** has the structure most consistent with the GC and mass spectral data exhibited by product **1**. This assignment implies the formation of the cubylcarbinyl radical during the reaction, which would be consistent with the proposed radical-based reaction pathway. Given the approximately equivalent rates of formation of **1** and **2** shown in Figure 4B, the hydroxyl rebound reaction is estimated to occur at about the known solution rearrangement rate of $3 \times 10^{10}$ s$^{-1}$, giving an apparent radical relaxation time of $2 \times 10^{-11}$ s.[3]

Table 2: Product Distributions of Methylcubane Hydroxylations Catalyzed by Different Enzyme Systems

| MMO (*Me. trichosporium*)[a] | MMO (*M. capsulatus*)[b] | P450s[c] |
|---|---|---|
| cubylmethanol 40% | cubylmethanol acetate 100% | cubylmethanol acetate 22% |
| methylcubanols 18% | | methylcubanol acetates 78% |
| **RP2** 42% | | |

[a] Distribution of products determined by integration of GC peak areas, assuming the same GC response factor for all products. [b] Reported data (*14*). [c] The average of values calculated on the basis of the normalized relative yields in Table 1 of ref *14*.

## DISCUSSION

The results of this study show that methylcubane, a radical clock substrate with a rigid structure, is hydroxylated in most, if not all, of the possible carbon positions when it is used as a substrate for MMO from *Me. trichosporium* OB3b. Moreover, a substantial yield of what appears to be a rearranged product is also observed. These results suggest that all C−H bonds in methylcubane are reactive toward the oxidizing species of **Q** and that the oxidation mechanism involves formation of a substrate intermediate with radical character. These are the expected results for a P450-like mechanism in which the first step in the attack on the substrate is postulated to be hydrogen atom abstraction which yields a substrate radical. However, past studies have shown that neither MMO isolated from *M. capsulatus* (Bath) nor P450 itself gives this product distribution with methylcubane (*14*) as illustrated in Table 2. The former enzyme was found to give only the side chain hydroxylation product without rearrangement, while P450s gave both side chain and cubyl hydroxylation products, but also no rearranged products. The distinct methylcubane oxidation reactions of these three enzymes, which catalyze the same or similar oxidation reactions of unactivated hydrocarbons, provide an opportunity to examine the factors which determine product distributions in this important enzyme class. These factors and the insights they provide into the mechanism of monooxygenase enzymes are discussed in the following sections.

*Basis for Differing Product Distributions for Monooxygenase-Catalyzed Reactions.* Monooxygenases that catalyze the oxidation of unactivated C−H bonds usually also promote oxidation of adventitious substrates, but generally at lower rates. P450 is considered to have an exceptionally wide substrate range (*37*). However, individual P450s from the large P450 family vary widely in their degree of specificity,

[3] The lifetime of the radical intermediate is determined as the reciprocal of the rate of its decay reaction which is the sum of the rate of rearrangement and the rate of rebound.

and most are fairly selective in their choice of substrate and position of oxidative attack. In contrast, MMO is a single enzyme with a broad substrate range. It is reasonable that this difference stems from the fact that the natural substrates for P450 are more complex, and thus more structural features are required in the active site to direct specific catalysis. Nevertheless, methanotrophs utilize only methane as a principal growth substrate, so selectivity must be achieved by some mechanism. Two possibilities are that the active site pocket selects methane on the basis of its size or that a mechanism is used that specifically enhances the rate of methane oxidation. The crystal structures of MMOH (*18*−*20*) and the fact that substrates much larger than methane are turned over by MMO suggest that the overall active site pocket is significantly larger than methane and unlikely to selectively exclude many other potential substrates. Likewise, it is difficult to conceive of a mechanism which would selectively oxidize the stable C−H bond of methane over the weaker bonds of similar hydrocarbons. Nevertheless, it is important to note that methane is not only the saturated substrate with the highest turnover number for MMO, but it also elicits the highest rate constant for **Q** decay at 5 °C. It is during this decay that the substrate oxidation reaction actually occurs (*21*). Thus, methane is oxidized faster and its product released more quickly than any other saturated substrate−product pair for MMO. A third possible basis for selectivity posits a combination of structural and kinetic influences. Subtle differences in accessibility to the diiron cluster for active site-bound substrates due to steric constraints may have large effects on the effective rate of the oxidation mechanism. Insight into this possibility can be gained from the current study of methylcubane oxidation in the context of previous studies.

*Evidence for Steering within the MMO Active Site.* Although methylcubane is apparently hydroxylated in all positions by MMO from *Me. trichosporium*, the distribution of products is not statistical. After correction for the relative C−H abundance, the side chain product cubylmethanol is formed approximately 5 times more often than methylcubanol,[4] despite the fact that cubyl C−H is inherently more reactive than side chain methyl C−H as indicated in the reaction of methylcubane with *tert*-butoxyl radical (*14*). Thus, the markedly nonstatistical distribution of products observed for MMO makes it very unlikely that the methyl C−H and cubyl C−H of methylcubane have equivalent access to the active site diiron cluster of MMO in the **Q** intermediate.[5] Similar regioselectivity has been observed in previous studies of other adventitious MMO substrates. For example, the product distribution for the substrate isopentane when turned over in the reconstituted *Me. trichosporium* MMO system in the absence of MMOB is essentially that expected on the basis of the bond strength of each type of C−H bond that is present. However, in the presence of MMOB, the distribution shifts dramatically to one which is nearly statistical and thus favors primary carbon hydroxylation (*13*) which cannot be explained on energetic grounds. It seems likely from this and other studies (*38*) that MMOB causes a conformational change in MMOH that alters the orientation in which adventitious substrates are presented to the reactive species, resulting in a shift in the product distribution. These considerations support the proposal that the active site structure might play a role in enhancing methane turnover by controlling the access of other potential substrates to the activated diiron cluster from the active site. Moreover, if the active site structure allows the smaller side chain carbon of methylcubane better access to the activated cluster in **Q**, then it is reasonable that the product distribution will shift toward cubylmethanol and potentially the rearranged products.

*Comparison of the Product Distributions from Different MMO Systems.* MMOs from two different sources have produced significantly different results with methylcubane (Table 2). If both of these results are correct, it suggests either that the two enzymes have different mechanisms or that they differ subtly in active site structure. Given the similarity in the spectral properties and kinetics of formation and decay of the reactive species in the two enzyme systems, it seems unlikely that they have different mechanisms. Although no major differences in structure are apparent, it is possible that the two MMOs exhibit minor differences in structure of the type responsible for the substrate steering effects described above. If so, then the bulk of the methylcubane cube, which we propose causes a bias against its hydroxylation in the *Me. trichosporium* MMO, may make it wholly unreactive with *M. capsulatus* MMO.

Another possibility for the difference in the results from the two systems stems from the facts that methylcubane has a very low turnover number and also has very low solubility in solution; this causes the product evolution to be relatively slow. Indeed, we experienced difficulty detecting products, especially the cubyl hydroxylation products, before we increased the preincubation time of the substrate in buffer with the enzyme to allow complete equilibration. Therefore, it is also possible that *M. capsulatus* MMO, which has a lower specific activity than *Me. trichosporium* MMO, did not yield enough product from the poorly positioned cube for detection. The absolute yield of all products from the two enzymes systems was also quite different. The reported yield for the *M. capsulatus* system was 0.5% (*14*), whereas our yield from the *Me. trichosporium* system was at least 5% and probably significantly greater.[6] The problem of the low yield from the *M. capsulatus* system may have been exacerbated by the procedure used for analysis in which the

[4] Reaction at the methyl group occurred approximately 10 times more often than at the cubyl C−H bonds, if both cubylmethanol and product **1** are considered to arise from a common intermediate.

[5] On the basis of the results of the oxidation of methylcubane by the *tert*-butoxyl radical, pure hydrogen abstraction chemistry should have favored cubyl hydroxylation products by 40/1 per hydrogen (*14*). For mechanisms other than hydrogen atom abstraction, the exceptional transition state stabilization responsible for the high reactivity of the cubyl C−H bonds is proposed to be lost (*14*). If so, they would be expected to react at a rate determined by their inherent bond dissociation energy, which is much higher than that of the methyl C−H bonds. Accordingly, the authors argued that the low level of production of methylcubanols stemmed from the use of a non-hydrogen abstraction mechanism by both P450 and MMO. This proposal, however, does not account for the observation that a much greater degree of cubyl hydroxylation is observed for P450 than for MMO-catalyzed oxidations despite the fact that the reactive **Q** species of MMO is apparently more reactive that the equivalent activated species of P450. It also does not recognize the fact that enzymes commonly control access to the reactive species so that all carbons of the substrate do not have equal access to the iron. Favored access to the reactive "Fe(V)"−oxo species for the side chain methyl group, together with rapid rebound chemistry, could result in the same product distributions that were observed in the previous study.

alcohol products were converted to acetate derivatives. This would have demanded more product for detection than our direct analysis method. Finally, the MMO from *Me. trichosporium* is maximally active at a temperature that is >15 °C lower than that from *M. capsulatus* or than was used for the *tert*-butoxyl radical oxidation study (*14*). Moreover, significant accumulation of products was possible in a few minutes using the enzyme system rather than in days as in the case of the *tert*-butoxyl radical system. Both of these factors may have allowed the significantly less stable rearranged products (*14*, *35*) to be more readily detected in the *Me. trichosporium* MMO system.

*Mechanistic Implications of the Rearrangement of Radical Clocks.* The observation of probable rearrangement in methylcubane oxidation by *Me. trichosporium* MMO suggests the formation of a substrate radical along the reaction pathway, thus supporting the "oxygen rebound" mechanism. However, the apparent lack of rearranged products reported for the *M. capsulatus* MMO was taken to suggest otherwise.[7] Many radical clock substrates have now been used in the investigation of the mechanism of MMO. These have inherent rearrangement rates ($k_r$) covering a wide range from that of 1,1-dimethylcyclopropane ($k_r = 10^8$ s$^{-1}$) (*10*) to that of phenyl-2-methylcyclopropane ($k_r = 10^{11}$ s$^{-1}$) (*11*). Of these clocks, only two, 1,1-dimethylcyclopropane and methylcubane, have exhibited significant amounts of apparent rearrangement, and then only with *Me. trichosporium* MMO. Mechanistic proposals accounting for the mostly negative results of radical clock studies often invoke concerted processes (*11*, *14*, *39*). These proposals were made under the assumption that only the reactive properties of the probe, i.e., the rate of the inherent rearrangement or the relative bond strengths of potential reactive positions, determines the outcome of the reaction. However, the fact that 1,1-dimethylcyclopropane rearranges during MMO-catalyzed oxidation (*10*) whereas the structurally and kinetically similar molecule 1,2-dimethylcyclopropane does not (*11*)[8] points to the inadequacy of such a consideration. This is further supported by the fact that the two molecules which apparently do rearrange have very different $k_r$ values ($10^8$ vs $10^{10}$ s$^{-1}$), suggesting that $k_r$ is not the sole determining factor in rearrangement. One feature that these two substrates do share in common is a bulky structure near the methyl group that is attacked by the enzyme. These results are consistent with the proposal that steric properties of the substrate play an important role in the reaction.

Past mechanistic studies have provided consistent indications of a discrete radical or cationic intermediate in the reaction mechanism of MMO (*15*, *29*). For instance, large deuterium KIE values were observed for the reaction of methane with intermediate **Q** (*31*), and partial inversion of the configuration of chiral ethane was observed to occur during oxidation to ethanol (*9*). However, the interpretation of each of these studies was complicated by an unusual aspect of the reaction. Thus, the KIE for the methane reaction with **Q** is much larger than the classical limit requiring that a tunneling or similar complex process be invoked. Similarly, the observed incomplete racemization of the product from chiral ethane requires that the rebound reaction occur on a time scale that is thought to be too fast for physical translocation of molecules in the active site. The range of results reported for radical clock substrates again provides evidence both for radical intermediates and for a complex process that is not completely understood. As described above in the analysis of the unexpected product distributions from methylcubane oxidation, one way to account for all of these results is to invoke a chemical mechanism which can be altered in its outcome by steric factors in the active site.

Mechanisms of this type have been proposed from advanced quantum chemical computational studies of MMO. Using density functional calculation methods, Siegbahn and Crabtree (*40*) were successful in independently predicting the same fundamental aspects of the structure of the iron cluster in **Q** as those determined directly from spectroscopic studies (*27*). Calculations to select the most likely structure of **P** as well as the most reasonable routes for the C−H bond breaking and oxygen insertion reactions themselves were also completed (*40*) and have since been further refined (*41*). These calculations suggest that C−H bond cleavage proceeds by hydrogen atom abstraction to yield a radical intermediate as proposed for P450 (*30*) and by us for MMO (*6*, *29*). However, this intermediate is further proposed to react very rapidly either to form an Fe−C bond (*40*) or to undergo a very rapid recombination reaction (*41*) due to the highly exothermic nature of this reaction for the specific intermediates that were formed.

Either of these mechanistic scenarios requires the radical to be very near the cluster and thus may depend critically on the size and bulk of the substrate. Such a precise orientation might provide an explanation for the apparent tunneling process that is unique to methane in the decay reaction of **Q**. Unlike other proposals (*14*, *42*−*44*), this model does not exclude the possibility of a linear C−H−O array in the transition state of the hydrogen abstraction process, a conformation that was strongly favored by KIE values observed in MMO-catalyzed reactions. This model would also explain the partial racemization observed in the chiral ethane reaction since either the formation of an Fe−C bond or exceptionally rapid rebound would limit rotation of the ethyl radical. For this model, the fundamental reaction mechanism is hydrogen atom abstraction for all saturated

[6] The yield is based on the total amount of products recovered relative to the amount of substrate utilized. Unfortunately, the substrate is very volatile, leading to error in measuring its loss. Also, the quantitation of products is based on the assumption that their GC response is the same, leading to another potential error. For incremental periods within the time course for *Me. trichosporium* MMO-catalyzed oxidation of methylcubane, where these errors are minimized, yields were typically greater than 40%. For measurements based on the amount of methylcubane present before the start of the reaction, the yield was always greater than 5%.

[7] The failure to observe rearranged products for MMO from *M. capsulatus* and P450s supported an intriguing proposal for monooxygenase chemistry in which the radical character required to explain some experimental results develops as a consequence of a nonsynchronous concerted reaction (*14*, *39*). In this model, differences in bond vibrational rates lead to a species with radical character and a lifetime in the 100 fs time range in the transition state ensemble. Because rearrangement of the cubylcarbinyl radical occurs on the comparatively slow 100 ps time scale, but rearranged products are still apparently observed in this study, the nonsynchronous concerted mechanism is unlikely in the case of *Me. trichosporium* MMO for methylcubane oxidation.

[8] 1,1-Dimethylcyclopropane was not tested with the *M. capsulatus* system. 1,2-Dimethylcyclopropane did not show rearrangement when reacted with either *M. capsulatus* (*11*) or *Me. trichosporium* MMO (Y. Jin and J. D. Lipscomb, unpublished results).

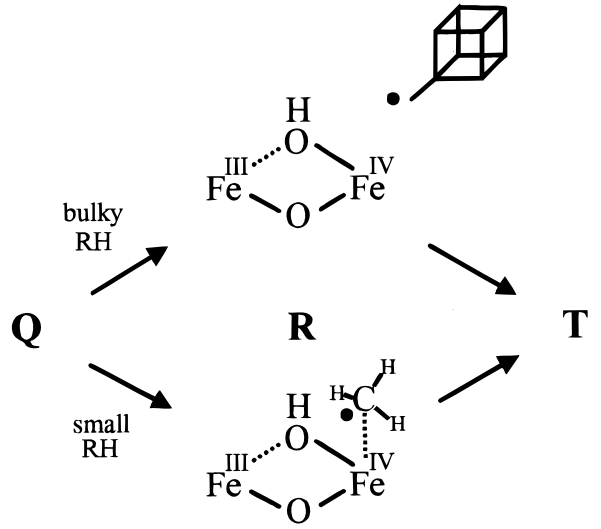


FIGURE 5: Hypothesis for the activation of C−H by MMO. The model is similar to the hydrogen atom abstraction mechanism proposed for P450 except that the lifetime of the substrate radical intermediate is influenced by the ability of the substrate to approach the diiron cluster in **Q**. Steric effects would limit access to the cluster by bulky substrates, whereas the small size of methane would allow it to reach the optimal position for rapid reaction.

substrates, but the lifetime of the radical intermediate would be quite variable depending upon the placement of the substrate determined by subtle steering within the active site, as illustrated in Figure 5. Small substrates would react similarly to methane with very fast rebound rates, while bulky substrates, which could not approach the active oxygen as closely, would react on a much longer time scale, allowing for radical rearrangement. Such a model would also help to reconcile the apparent variability observed in radical clock studies. For clocks, such as 1,2-dimethylcyclopropane, which could approach the cluster closely and thus undergo rapid oxygen rebound chemistry, electron density would be prevented from migrating into the cyclic structure as required for opening, resulting in no rearrangement. On the other hand, clocks with "bulky" C−H, e.g., 1,1-dimethylcyclopropane or methylcubane, that could not approach the cluster as closely would have longer-lived free radical intermediates that would have a chance to rearrange before the rebound reaction. In this scenario, the bulk of the probe plays a more important role than its inherent rate of rearrangement in determining whether the isomerization would take place.

Structures for the MMOH active site are only available for the diferric and diferrous states in the absence of MMOB (*18*−*20*). Thus, the possible origin of substrate steering effects is quite speculative. However, the only groups which protrude into the potential substrate pocket are the iron ligands Glu114, Glu209, and Glu243, which frame the approach to the cluster. These groups may provide some selection against bulky molecules.

*Structural Basis for Methylcubane Rearrangement during Oxidation.* In the mechanistic scheme for methylcubane side chain oxidation illustrated in Figure 6, the cubylcarbinyl radical would form and undergo rapid rearrangement. The fairly rigid geometry of the cube structure of methylcubane would probably be retained during the rearrangement since it happens at such a fast rate. Thus, the subsequent collapse with the iron-bound hydroxyl moiety would occur regio-

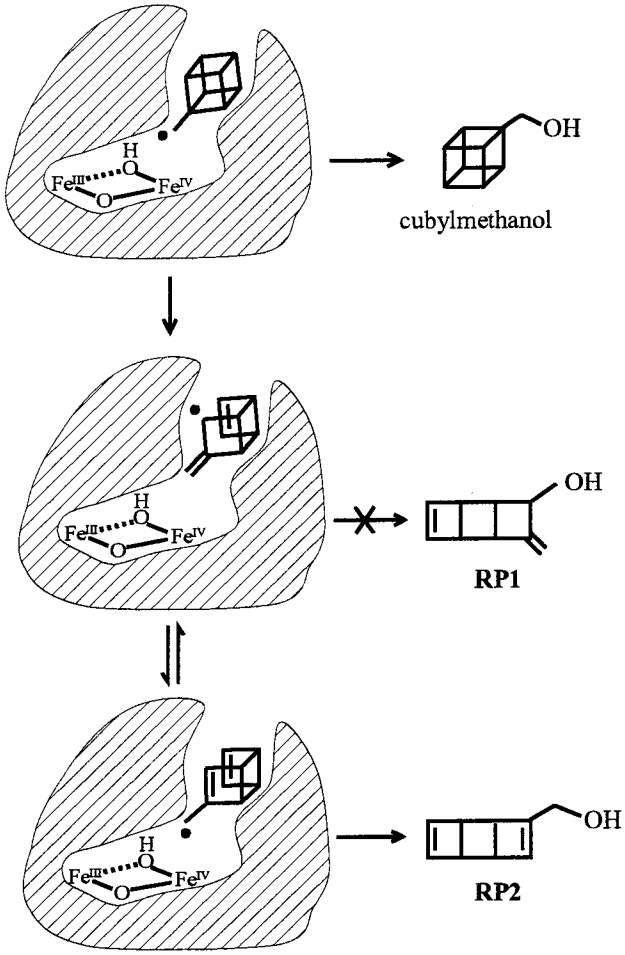


FIGURE 6: Hypothetical model accounting for the production of only **RP2** as the rearranged product in the reaction of methylcubane with MMO from *Me. trichosporium* OB3b.

selectively. This would favor the processes in which one of the Kekulé isomers, apparently the one that has the electron density at the original methyl carbon, recombines with the metal-bound hydroxyl group to form the hydroxylation product, **RP2**. In the absence of such steric effects, the other possible rearranged product stemming from a cubylcarbinyl radical would be expected, but is not observed.

*Summary*. Methylcubane is an excellent probe of the mechanism of MMO because it reveals selectivity where it was not expected. Specifically, it shows that (i) the MMO active site can distinguish between the cubane structure and the side chain in the molecule and (ii) the active sites of two very similar MMO hydroxylases can apparently yield different product distributions despite apparently similar or identical chemical mechanisms. Together, these observations suggest that subtle structural features of the active site are capable of altering the approach of substrates to the activated oxygen species in **Q** and that this critically affects the reaction outcome and rate. A mechanism in which a species with the formal oxidation state of Fe(V)═O initiates the oxidation reaction by hydrogen atom abstraction would account for both the apparent rearrangement of one of the methylcubane products and the rapid reaction rate of methane, the hydrocarbon least likely to be affected by steric considerations in the active site.

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