Cationic Species Can Be Produced in Soluble Methane Monooxygenase-Catalyzed Hydroxylation Reactions; Radical Intermediates Are Not Formed

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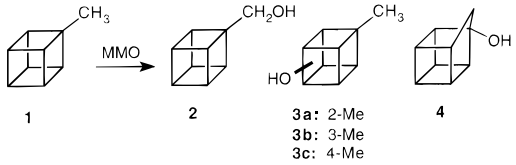
Soluble methane monooxygenase (MMO) systems from methanotrophic bacteria contain hydroxylase enzymes (MMOH) that have a diiron moiety at the active site. The MMOH enzymes efficiently oxidize methane to methanol, one of the more remarkable reactions in nature. Several intermediates in the catalytic cycle of MMOH accumulate and have been characterized spectroscopically.[2] The mechanisms of catalytic hydroxylation of unactivated C−H bonds by these enzymes are not fully understood, however, and remain a subject of considerable research effort. The MMO hydroxylation reactions could be related to hydroxylations by cytochrome P450 enzymes.[3]

Various mechanistic probes have been employed in studies of the two well-characterized MMO systems, those from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b. The results from oxidations of chiral (by virtue of isotopic substitution) alkanes[4,5] and hypersensitive cyclopropane-based probes[6,7] are in general agreement that the "lifetimes" of putative radicals in MMO hydroxylation are too short for true intermediates and thus implicate insertion reactions. In a study reported by our groups in 1996, the regiochemistry of reactions of methylcubane with the *tert*-butoxyl radical, a cytochrome P450 enzyme, and the sMMO system from *M. capsulatus* (Bath) were compared.[8] We reported that, whereas P450 oxidized all CH positions to give four alcohol products, the MMO oxidation gave only cubylmethanol, and we proposed an insertion mechanism for this oxidation. Recently, it was reported[9] that the *M. trichosporium* OB3b sMMO system oxidized methylcubane at all positions and that the major alcohol product derived from rearrangement of the cubylcarbinyl radical, thus implicating a radical-based mechanism.

We have now reinvestigated methylcubane oxidation by both sMMO systems. All positions of this substrate are indeed functionalized by both sMMOs, and the observed[9] rearranged alcohol is produced in both cases. That rearranged product, however, is shown here to be the known compound 1-homocubanol, which derives not from a radical intermediate but from a *cationic* rearrangement process. In addition, a hypersensitive cyclopropane-based mechanistic probe designed to distinguish between "radical" and "cationic" species was successfully oxidized by the *M. capsulatus* (Bath) MMOH, and a cationic rearrangement product was detected as a minor product. The results clearly implicate a "cationic" component in MMO hydroxylation reactions as previously suggested,[10] and they support the conclusion that more than one species can effect oxidation in sMMO systems.[2]

Oxidations of methylcubane (**1**) were performed with the MMO systems from *M. capsulatus* (Bath) and *M. trichosporium* OB3b.[11] GC and GC-MS analyses of the product mixtures revealed the presence of five alcohol products from the *M. c.* (Bath) MMO oxidation (Supporting Information). The same products were formed in lower yields in the *M. t.* OB3b MMO oxidation, although the GC traces from these reactions were complicated by the presence of traces of compounds from the enzyme mixture. Oxidation of methylcubane by the purified cytochrome P450 isozyme CYP2B1[8] in a control experiment gave a clean product sample containing four of the five alcohol products. Cubylmethanol (**2**) was identified by comparison of the GC retention time and mass spectral fragmentation pattern to those of the known compound.[12] The three methylcubanol products (**3**) were identified by their mass spectral fragmentation patterns.[9]



The fifth alcohol product from MMO oxidations, the major product, was not formed in detectable amounts in the P450-catalyzed oxidation. This product is the rearranged compound 1-homocubanol (**4**) as determined by comparison of its GC retention time and mass spectral fragmentation pattern (Supporting Information) to those of an authentic sample.[13] The mass spectrum of **4** is the same as that shown in Figure 4C of ref 9, and there is little doubt that this product is the one ascribed to a radical rearrangement.[9] A control experiment showed that alcohol **2** does not convert to **4** during steady-state hydroxylation of acetonitrile, a good substrate for MMO.[14] To confirm product identities, a mixture of products from *M. c.* (Bath) was treated with acetic anhydride and pyridine to give acetates that were characterized by GC and GC-MS. We found five acetates with GC retention times and MS fragmentation patterns matching those of the known[8,15] acetates from products **2** and **3** and the prepared acetate from **4**.[16]

The production of 1-homocubanol in the MMO oxidations is inconsistent with formation of a radical intermediate. The cubylcarbinyl radical ring opens by a series of bond cleavage reactions that destroy the cube skeleton.[12,17] Ring expansion of the cubylmethyl system to the homocubyl system is only known for cationic rearrangements.[12,13,18]

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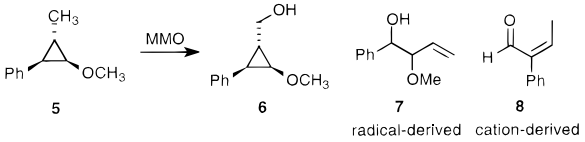
In a typical experiment, the GC (flame-ionization) peak areas of the alcohols obtained from the *M. c.* (Bath) MMO oxidation of methylcubane were in the ratio 2:3:5 (**2**, **3**, **4**), and product **4** was the major one with *M. t.* OB3b as previously reported.[9] The methylcubanols and 1-homocubanol are unstable compounds, however, and a control reaction containing a small amount of cubylmethanol (**2**) showed that this compound was consumed by MMO. The product instabilities might account for the differences in the present results from those we previously found with *M. c.* (Bath).[8] These instabilities require that the product ratios be considered as *qualitative* rather than quantitative measures.

An alternative molecular architecture that can distinguish between radical and cationic intermediates is afforded by the hypersensitive cyclopropane-based probe **5**. The cyclopropylcarbinyl radical from **5** rearranges to give products derived from a benzylic radical, but the cyclopropylcarbinyl cation ring opens to the methoxy-substituted cation (an oxonium ion).[19,20] In the context of hydroxylation reactions, alcohol **7** is produced from radicals, whereas aldehyde **8** is formed from cations via hydrolysis of the initially formed hemiacetal and isomerization of the β,γ-unsaturated aldehyde in buffer. We attempted to oxidize the *tert*-butoxy analogue of probe **5** with the *M. c.* (Bath) sMMO system, but that compound was an inhibitor and not a substrate.[7]



Probe **5** is a substrate, albeit a relatively poor one, for the sMMO from *M. c.* (Bath). Oxidations of **5** gave the unrearranged alcohol **6** as the major product and both radical-derived (**7**) and cation-derived (**8**) rearrangement products. Two diastereomers of **7** are possible, but only one was detected. The product alcohols from the MMO oxidation were identified by comparisons of GC retention times and mass spectral fragmentation patterns to those of authentic samples.[21] The yields of alcohols were small, rendering quantitation difficult, but the product ratios from four oxidations of **5** were 80:14:6 for **6**, **7**, and **8**, respectively.

The accumulated results of probe studies of MMO hydroxylations permit the firm conclusion that *no radical intermediates are formed*. No radical rearrangement products are found from methylcubane. From the product ratio and rate constant for ring opening of the cyclopropylcarbinyl radical derived from probe **5**,[20] the "radical" lifetime was computed to be 250 fs, corresponding to a capture rate constant of 4×10^{12} s^{-1}. The small amounts of inversion found in MMO hydroxylations of chiral ethane[4,5] and butane[5] require that the lifetimes of the "radicals" be on the order of 100−200 fs. A variety of hypersensitive cyclopropane-based radical probes that cannot distinguish between radical and cationic rearrangements previously gave little or no rearrangement products[6,7] requiring that the "radical" lifetimes be 250 fs or less. The lifetimes being measured are those of transition states.

In contrast to the firm conclusion regarding the absence of radical intermediates, the detection of cationic rearrangement products from both methylcubane and probe **5** permits only qualitative statements. The lifetimes, and even the identities, of these species cannot be determined. A similar conclusion resulted from *M. t.* OB3b sMMO oxidation of 1,1-dimethylcyclopropane which gave some 1-methylcyclobutanol product.[10] Candidates for the cationic species are protonated alcohols.

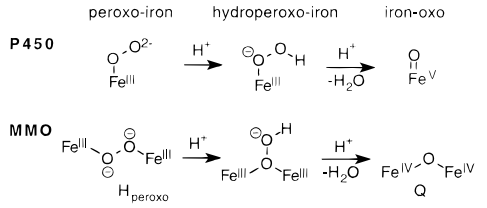
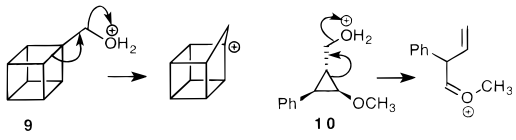


Figure 1. Possible analogous intermediates produced in cytochrome P450- and MMOH-catalyzed oxidations. The porphyrin ring of P450 and the surrounding protein of MMO have been removed. The intermediates for P450 and the hydroperoxo complex for MMO are putative. The structures shown are speculative.

The oxidations catalyzed by the sMMO and cytochrome P450 enzyme systems have been compared;[3] the intermediate oxidants inferred for P450[21−23] may be related to the spectroscopically observed intermediates found for MMO (Figure 1). Two electrophilic oxidant forms, presumed to be the hydroperoxo−iron complex and the iron−oxo, are implicated in the P450 reactions.[22] The hydroperoxo−iron species apparently hydroxylates by inserting "OH^+" into C−H bonds to give protonated alcohols, whereas the iron−oxo inserts an oxygen atom.[21] Single turnover experiments with MMOH from *M. c.* (Bath) indicate that either H_{peroxo} or, more likely, a hydroperoxo species derived therefrom by protonation is an active oxidant that can epoxidize propene. This oxidant did not hydroxylate saturated hydrocarbons methane, ethane, or propane, however.[2] If the hydroperoxo species in MMOH were able to hydroxylate the substrates studied here, then, by analogy to P450, the first-formed products would be the protonated alcohols **9** and **10** produced by insertion of "OH^+" into C−H bonds.



In summary, the rearrangement product from sMMO-catalyzed hydroxylation of methylcubane is 1-homocubanol, formed via a cationic rearrangement, and production of a cationic species was also demonstrated in sMMO hydroxylation of probe **5**. Mechanistic studies of sMMO hydroxylations involving chiral alkanes,[4,5] hypersensitive cyclopropane-based probes,[6,7] methylcubane, and probe **5** provide compelling evidence that true radical intermediates are not produced in these reactions. The implication of a cationic component in MMO hydroxylations complicates the mechanistic picture, but it reinforces some similarities between P450 and MMO hydroxylation reactions.[3] Future mechanistic studies of MMO hydroxylations of alkanes will focus on further assessment of the oxidizing properties of the H_{peroxo} and Q intermediates, both of which can be observed.

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Supporting Information Available: GC traces of products from reactions of **1** with enzymes and mass spectra of **4** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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