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ORIGINAL ARTICLE

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Desaturation reactions catalyzed by soluble methane monooxygenase

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Abstract Soluble methane monooxygenase (MMO) is shown to be capable of catalyzing desaturation reactions in addition to the usual hydroxylation and epoxidation reactions. Dehydrogenated products are generated from MMO-catalyzed oxidation of certain substrates including ethylbenzene and cyclohexadienes. In the reaction of ethylbenzene, desaturation of ethyl C-H occurred along with the conventional hydroxylations of ethyl and phenyl C-Hs. As a result, styrene is formed together with ethylphenols and phenylethanols. Similarly, when 1,3- and 1,4-cyclohexadienes were used as substrates, benzene was detected as a product in addition to the corresponding alcohols and epoxides. In all cases, reaction conditions were found to significantly affect the distribution among the different products. This new activity of MMO is postulated to be associated with the chemical properties of the substrates rather than fundamental changes in the nature of the oxygen and C-H activation chemistries. The formation of the desaturated products is rationalized by formation of a substrate cationic intermediate, possibly via a radical precursor. The cationic species is then proposed to partition between recombination (alcohol formation) and elimination (alkene production) pathways. This novel function of MMO indicates close mechanistic kinship between the hydroxylation and desaturation reactions catalyzed by the nonheme diiron clusters.

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Abbreviations *MMO*: soluble methane monooxygenase · *MMOH*: hydroxylase component of methane monooxygenase · *MMOB*: B component of methane monooxygenase · *MMOR*: reductase component of methane monooxygenase · ***O***, ***P****, ***P***, ***Q***, ***R***, ***T***: intermediates of the MMO catalytic cycle · *RNR-R2*: R2 subunit of ribonucleotide reductase · *Δ9D*: stearoyl-acyl carrier protein Δ^9-desaturase · ***X***: intermediate X of the RNR-R2 catalytic cycle

Introduction

The soluble methane monooxygenase (MMO) system is remarkable in that it can catalyze the cleavage of the stable C-H bond in methane, react with an exceptionally broad range of substrates, and promote the formation of many chemically distinct types of products [1, 2]. For example, the substrates for MMO beyond methane include saturated, unsaturated, linear, branched, cyclic, aromatic, heterocyclic, and halogenated hydrocarbons. Hydroxylation, epoxidation, *N*-oxide formation, and dehalogenation are among the many different types of reactions catalyzed by the enzyme [3, 4, 5]. This extraordinary catalytic chemistry of MMO is carried out at a nonheme diiron cluster in the active site located in the hydroxylase component (MMOH) [6, 7]. The other two components of the system, the "B" component (MMOB) and reductase (MMOR), serve important regulatory and electron transfer roles, respectively [8, 9, 10].

Like the heme center commonly found in oxygenases such as cytochrome P450, the diiron cluster harbored in MMOH is utilized in a number of enzymes for diverse functions. This class of enzymes also includes the R2 component of ribonucleotide reductase (RNR-R2) [11], the plant fatty acid desaturases, e.g. stearoyl-ACP Δ^9-desaturase (Δ9D) [12], and other bacterial hydrocarbon

monooxygenases [13, 14, 15, 16, 17]. The roles of the metal centers and the reactions carried out by these enzymes are different. The diiron cofactor of RNR-R2 is involved in the generation of a radical on a nearby tyrosyl residue which is ultimately used in the dehydroxylation reaction of ribonucleotides [18, 19, 20]. The metal center in desaturases, on the other hand, is responsible for the oxidative desaturation of the fatty acids [21, 22, 23]. In contrast to MMO, neither of these enzymes incorporates oxygen into substrates as a part of their normal reaction chemistry, even though O_2 is activated as a requirement for catalysis. Nevertheless, extensive spectroscopic and crystallographic studies have revealed that the diiron centers of the O_2-activating enzymes have very similar ligation environments [6, 7, 12, 24, 25], suggesting a common mechanistic theme [2, 26].

The mechanisms of many diiron enzymes are currently being investigated. In the case of MMO, detailed transient kinetic studies have shown that the catalytic cycle proceeds through several key intermediates, including a peroxo or hydroperoxo adduct of the diiron cluster termed **P** and a unique dinuclear Fe(IV) species termed **Q** (Fig. 1) [1, 27, 28, 29]. **Q** contains the activated oxygen species and attacks the stable C-H bond in the substrate. Similar chemistry is believed to occur during the reaction of RNR-R2 with O_2 to produce the reactive intermediate **X**, which is responsible for the formation of the tyrosyl radical [20, 30]. Although the diiron centers in these key reactive intermediates of the reactions are in different oxidation states, Fe(IV)Fe(IV) and Fe(IV)Fe(III) for **Q** [31] and **X** [32], respectively, one can easily envision how **X** could be generated from a **Q**-like intermediate in RNR-R2 by one-electron reduction. Indeed, a one-electron reduction must be an important part of the RNR-R2 reaction mechanism because the end product of RNR-R2 catalysis is a radical, whereas those of MMO have singlet ground states. Spectroscopic studies of **Q** and **X** have shown similar features in the core structure of the diiron centers [33, 34]. In addition, peroxo-diiron intermediates similar to **P** of the MMO catalytic cycle were detected with mutants of RNR-R2 [35]. Although less information is available regarding the catalysis of Δ9D, a **P**-like intermediate was observed from the reaction of the chemically reduced enzyme with O_2, and a high-valent species parallel to **Q** or **X** was proposed to be responsible for its reactivity [36, 37].

If the diiron oxygen-activating enzymes do share a common mechanism through at least part of their catalytic cycles, it would seem possible that they would be able to catalyze reactions typical of other members of the class when presented with unusual substrates or reaction conditions. For example, it has been shown that site-directed mutagenesis of residues in the vicinity of the diiron cluster of RNR-R2 enables the enzyme to function as an oxygenase [38].

Here we report a novel function for the diiron cluster of MMO: its ability to catalyze desaturation reactions. The reactions of several substrates with the MMO enzyme from *Methylosinus trichosporium* OB3b have been examined under different conditions. The results show that the MMOH-catalyzed oxidations of certain substrates generate dehydrogenated products in addition to the products from conventional hydroxylation and epoxidation reactions. When considered in the light of the structurally related fatty acid desaturases, the results of this study may provide insight into the mechanistic kinship between hydroxylation and desaturation reactions catalyzed by the nonheme diiron clusters.

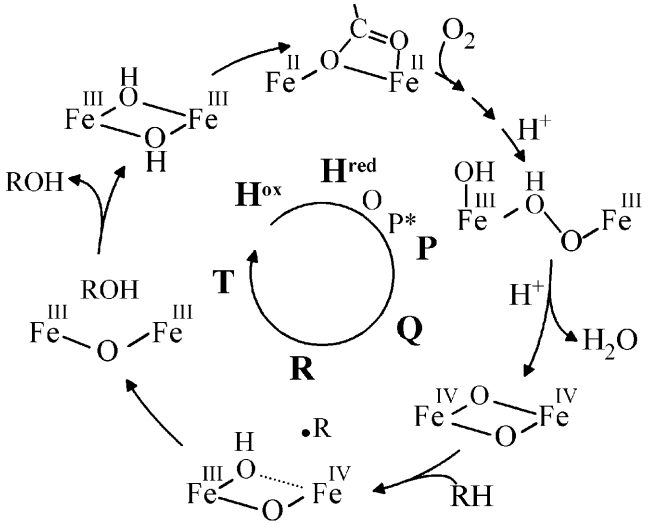

Fig. 1 Key intermediates in MMOH catalytic cycle. H^{red}, the diferrous state of MMOH; H^{ox}, the diferric state of MMOH; ***O***, ***P****, ***P***, ***Q***, ***R***, ***T***, intermediates of the MMO catalytic cycle. The structures shown represent our best current understanding based on crystallographic (H^{ox} and H^{red}) and spectroscopic studies. Intermediate **R** has not been directly observed, but its formation is suggested base on diagnostic chemical reactions indicating a radical intermediate

Materials and methods

Materials

The components of MMO were purified to homogeneity from *M. trichosporium* OB3b. Details of the growth of the bacterial strains, protein purification, and activity assay procedures were published previously [39, 40]. MMOH used in this work exhibited specific activity in the range of 600–1000 nmol min^{-1} mg^{-1} at 23 °C for furan turnover. All reagents were of the highest grade available and obtained from Sigma, Aldrich, or EM Scientific. Water was deionized and further purified using a multi-stage Millipore water purification system. The inert gas used for anaerobic sample preparation was O_2-free argon that had been passed through an activated copper oxygen-scrubbing trap (BASF) at 140 °C. The buffer for all experiments was 50 mM MOPS, pH 7.5.

MMO-catalyzed reactions

NADH-coupled reactions

Experiments were carried out as previously described [41]. In a typical reaction, concentrated MMO enzyme components were

added to a reaction vial containing oxygenated buffer to reach the desired final concentration (e.g. 30 μM for MMOH, 60 μM for MMOB and MMOR). Substrates were added neat or from stock substrate saturated solution made by dissolving the substrate in oxygen-saturated buffer. The reaction mixture was allowed to equilibrate for 15 min on ice and then warmed to 30 °C. The reaction was initiated by the addition of 5 μL 350 mM ethanol-free NADH and allowed to proceed at 30 °C in a temperature-controlled shaker.

H_2O_2-coupled reactions

In a reaction vial, stock H_2O_2 solution (quantitated by optical absorbance at 240 nm, $\epsilon = 43.6\ M^{-1}\ cm^{-1}$) was diluted to 100 mM in buffer. Neat substrate or stock substrate solution was added. The reactions were initiated by the injection of concentrated MMOH solution (final concentration 150 μM).

Single-turnover reactions

MMOH (350 μM, 700 μM active site) and methylviologen (100 μM) were combined in a reaction vial. The vial was sealed and the solution was made anaerobic. Sodium dithionite was added to stoichiometrically reduce the enzyme (2 reducing equivalent/active site). Reduction was confirmed by the stable appearance of blue color from the reduced methylviologen. The reaction was initiated by the injection of an equal volume of oxygenated substrate solution ($[O_2] \approx 1$ mM) containing 700 μM MMOB, followed by exposing the reaction solution to air.

Organic syntheses

3,4-Epoxycyclohexene [42], 4,5-epoxycyclohexene [43], and 2,4-cyclohexadienol [44] were prepared according to the designated literature procedures with minor changes as described below. NMR spectra were obtained using Bruker (^{1}H, 300 MHz and 500 MHz) spectrometers.

3,4-Epoxycyclohexene

At 0 °C, 1,3-cyclohexadiene (1.0 g) was mixed with sodium carbonate (6.5 g) and water (1 mL) in 25 mL of dichloromethane. To the mixture, 70% of *m*-chloroperbenzoic acid in 15 mL methylene dichloride was added dropwise. After the addition was complete, the mixture was left at room temperature for 20 min. The solution was filtered and then dried with anhydrous $MgSO_4$. 3,4-Epoxycyclohexene was obtained by distillation as a colorless liquid. ^{1}H NMR (CD_2Cl_2): δ 1.5–2.2 (m, 4H), 3.1 (m, 1H), 3.3 (m, 1H), 5.9 (m, 2H).

4,5-Epoxycyclohexene

About 8 g of 1,4-cyclohexadiene in dichloromethane (140 mL) containing anhydrous sodium carbonate (14 g) was stirred at room temperature while peracetic acid (7.8 mL, 1 equiv), previously saturated with sodium acetate trihydrate, was added. After the addition was complete, the stirring was continued for 15 h. Then, the mixture was diluted with 200 mL of water and the dichloromethane layer was washed successively with aqueous sodium carbonate and brine before being dried (by $MgSO_4$). Final distillation yielded about 6 g of 4,5-epoxycyclohexene as a colorless liquid. ^{1}H NMR (C_6D_6): δ 1.8–2.3 (m, 4H), 2.8 (s, 2H), 5.2 (s, 2H).

2,4-Cyclohexadienol

Under Ar, MeLi (52 mL) was added dropwise into a flask in an ice bath containing 4,5-epoxycyclohexene (4.8 g) and ether (40 mL). After the addition, the mixture was warmed to room temperature while the evolution of gas was monitored. At 15 min after gas formation had ceased the mixture was hydrolyzed with 8 mL of 5% NaOH. The aqueous phase was separated out and washed with NaCl-saturated water twice. After drying over K_2CO_3, the solution was evaporated and distilled. Distillation at reduced pressure afforded 2 g of the desired compound as a pale yellow liquid. ^{1}H NMR (C_6D_6): δ 1.2 (s, 1H), 2.0–2.4 (m, 2H), 4.0 (s, 1H), 5.5–5.8 (m, 4H).

Analysis of product by GC and GC/MS

To analyze the oxidation products, the reaction was stopped by the addition of an equal volume of an organic solvent that contained a small amount of an internal standard for GC analysis. Chloroform was the primary solvent used in most experiments, but for each substrate, dichlorobenzene was used at least once as the alternative quenching solvent. This was to ensure that the solvent peak of chloroform masked no product with shorter retention time since dichlorobenzene has a clean baseline in the low retention time region. Vigorous mixing of the organic solvent with the reaction solution was achieved using a vortex mixer, and the mixture was then centrifuged for 1–2 min. About 1–2 μL of the organic extract was injected into the gas chromatograph without further treatment. GC analysis was conducted on a Hewlett-Packard (HP) Model 5890 gas chromatograph equipped with an FID detector and a capillary DB-1 column. To obtain the time course of the formation of products, samples were withdrawn at desired time points. The formation of products was referenced to the GC trace of the reaction mixture extracted with chloroform prior to addition of NADH. Products were identified and quantitated by comparison with authentic standards. When authentic materials were not available, relative yields of product were determined by integration of GC peak areas with the assumption of equal GC response factors.

For 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 to a Kratos MS-25 spectrometer (EI, 70 eV).

Results

Oxidation of ethylbenzene

The reaction of ethylbenzene with the reconstituted MMO (all three MMO components plus NADH and O_2) from *M. trichosporium* OB3b generated multiple products (Fig. 2 and Table 1), as revealed by GC analysis of the reaction mixture. By comparison with the GC elution properties of the authentic standards, *p*-ethylphenol and 1-phenylethanol are found to be the major products of the reaction. Together with 2-phenylethanol, they are the expected products from the conventional hydroxylation reactions at the phenyl and the ethyl C-H positions of the substrate. The system exhibited a preference for secondary C-H hydroxylation at the ethyl group but still yielded some of the primary C-H hydroxylation product, 2-phenylethanol. Significantly, styrene and styrene epoxide were also among the products, with the latter appearing to be the further oxygenation product from styrene. This is indicated by the correlation between the decrease of styrene and the increase of styrene epoxide as the reaction proceeds

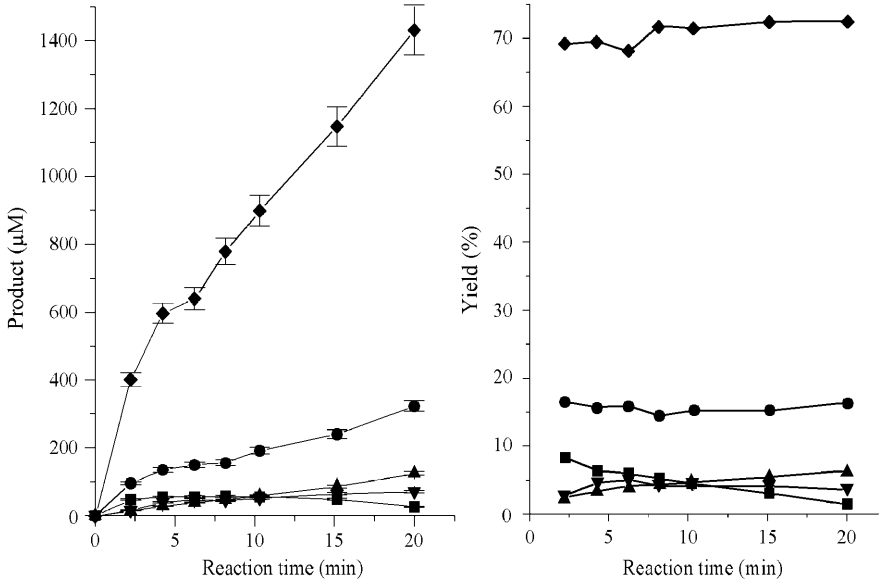

Fig. 2 Time course for the formation (*left*) and relative yield (*right*) of products from oxidation of ethylbenzene catalyzed by reconstituted MMO: *p*-ethylphenol (◆), 1-phenylethanol (●), styrene epoxide (▲), 2-phenylethanol (▼), and styrene (■)

(Fig. 2, left), while the total combined yield of the two remained the same relative to those of the other products[1]. The distribution of the products, with the exception of styrene and styrene epoxide, was observed to be stable for at least 20 min during the reaction (Fig. 2, right). The identification of the products was further confirmed by mass spectral analysis.

Several control reactions were conducted to more firmly establish that the observed production of styrene and styrene epoxide results from the desaturation of ethylbenzene by the action of MMO. The dependence of the formation of styrene on MMOH reactivity and concentration was tested and confirmed. For example, for the NADH-coupled reaction, no product was formed before the addition of NADH or when denatured MMOH was used. Increasing the amount of MMOH used for the reaction linearly increased the amount of styrene formed. The possibility that styrene may arise from dehydration of initially formed alcohols during the reaction was excluded. Under the same experimental conditions, both 1- and 2-phenylethanol were found to be chemically stable with no occurrence of the dehydration reaction. Furthermore, when they were subjected to the reaction with MMOH, no styrene or styrene epoxide was detected. This shows that styrene generated from the oxidation of ethylbenzene did not arise from nonenzymatic or enzymatic dehydration of its initial alcohol products under the reaction conditions employed.

The reaction of ethylbenzene with MMOH was also investigated under peroxide-shunt and single-turnover reaction conditions. In the peroxide-shunt system, only MMOH and H_2O_2 are required for catalysis, although the other MMO components can be added to evaluate their effects on the reaction outcome [45, 46]. The single-turnover system was developed to search for the reaction intermediates shown in Fig. 1. In this system, reduced MMOH, MMOB, and O_2 are generally present, although slow single turnover is possible in the absence of MMOB [9, 27]. The influence of reaction conditions in terms of the systems used and protein components present on product formation from ethylbenzene oxidation was studied in detail, and the results are listed in Table 1. In all cases, styrene was detected among products, although there are differences in the types and distributions of the other products. The same set of products except for styrene epoxide was produced from the single-turnover reactions as from the NADH-coupled multiple-turnover reactions. This is consistent with the styrene epoxide formed in the latter being the secondary oxidation product of styrene. Unlike these two reaction systems, the H_2O_2-coupled system generated phenols at the *ortho* and *meta* positions in addition to the *para*-ethylphenol and produced little hydroxylation at the primary carbon of the ethyl group. In some cases, phenylethanone was formed[2]. The presence of MMOB in the H_2O_2-coupled reaction system also showed profound effects on product distribution (Table 1), whereas that of MMOR or mannitol (as a hydroxyl radical scavenger) did not induce any significant change in the relative yield of styrene or product distribution (data not shown). This strongly supports our contention that the peroxide-coupled reaction is occurring in the active center of MMOH because MMOB would not be expected to have any direct effect on the chemical reaction in solution. However, it is known to bind specifically to

[1]We have observed that epoxidation of an unsaturated substrate catalyzed by MMO usually occurs at a higher rate than hydroxylation

[2]Phenylethanone is speculated to be the product from secondary oxidation of ethylbenzene. It is interesting to note that no phenylethanone was generated when a stoichiometric amount of MMOB is present

Table 1 Product distributions of the oxidation reactions of ethylbenzene catalyzed MMOH

Reaction system[a]	Distribution of products (% of total)[b]						
	Ethylphenols			Phenylethanol		Styrene plus styrene epoxide[c]	Phenylethanone
	p-	*m*-	*o*-	1-	2-		
NADH coupled							
H + 0B + 2R	31	nd	nd	59	10	< 0.5	nd
H + 0.2B + 2R[d]	71	nd	nd	16	4	9	nd
H + 0.6B + 2R	70	nd	nd	16	5	9	nd
H + 2B + 2R	71	nd	nd	16	4	9	nd
Single turnover							
H + 2B	71	nd	nd	16	5	5	nd
H_2O_2 coupled							
H + 0B	24	7	5	45	nd	4	15
H + 0.1B	27	10	5	43	nd	3	3
H + 2B	45	11	6	32	nd	6	nd

[a]The experimental conditions for the three different reaction systems are as described in the Materials and methods section. The protein components of MMO present in the reaction mixture were listed in relation to MMOH (mol/mol). H, B, and R refer to MMOH, MMOB, and MMOR. Because MMOH has two active sites per molecule, H + 2B + 2R is a stoichiometric complex

[b]Relative yields of products were determined by comparison with the authentic materials except that equal GC response factors were assumed for the different ethylphenols. The error for data from single turnover experiments and the H_2O_2-coupled MMOH reactions in the presence of 2 equiv of MMOB is 10%, and for the rest it is 6%. nd, not detected

[c]For NADH-coupled reactions; the numbers reflect the sum of styrene and styrene epoxide formed

[d]We have observed previously that substoichiometric MMOB is sufficient to induce the complete shift in product distribution, suggesting a hysteretic interaction with MMOH [46]

MMOH, altering its active site structure and the product distributions observed from other substrates [46, 47].

The use of ethylbenzene as a substrate also permits the comparison of the rates between phenyl and ethyl hydroxylations. The NADH-coupled and single-turnover reactions of MMOH showed a strong bias toward hydroxylation at the *para* position of the benzene ring, whereas for the H_2O_2-coupled MMOH system the hydroxylation at the secondary carbon of the ethyl group is preferred. More importantly, the changes in reaction conditions also caused changes in the relative amount of desaturation versus hydroxylation at the same position.

Oxidations of cyclohexadienes

Three product peaks were detected from the GC analysis of the 1,3-cyclohexadiene oxidation catalyzed by MMO. One product was readily identified as benzene, the desaturation product, using an authentic standard. To identify the other products, we synthesized two possible candidates, 3,4-epoxycyclohexene and 2,4-cyclohexadienol, expected from epoxidation and hydroxylation of the substrate, respectively. GC-MS analysis showed that these were the other two products obtained. Thus, this substrate appears to be oxidized in three types of reactions: epoxidation, hydroxylation, and desaturation (Table 2).

Similarly, three products were generated from the oxidation of 1,4-cyclohexadiene catalyzed by MMO (Table 2). Again, benzene was detected from the reaction. Using synthesized material as standard for GC/MS analysis, 4,5-epoxycyclohexene was also found to be among the products. The third product was assigned to be 2,5-cyclohexadienol, which is a logical candidate based on comparison with the reaction of 1,3-cyclohexadiene. This assignment is also supported by the fact that its MS spectrum shows a fragmentation pattern (*m*/*z* 95, 81, 77, 67, 65, 55, 51, 41, 39) that is almost identical to that of 2,4-cyclohexadienol (*m*/*z* 95, 81, 78, 67, 63, 51, 39). Unfortunately, because there is no known procedure for the synthesis of 2,5-cyclohexadienol, final confirmation of the identity of the third product cannot be made.

The distributions of products from the reactions of the cyclohexadienes were stable for at least the first 5 min of the reaction. Control experiments showed that 3,4- and 4,5-epoxycyclohexenes are stable compounds under the condition of the experiments. It has been reported that in acidic solution they undergo hydrolysis, producing diols [48]. Evidence for diols was searched for in GC and GC-MS analyses of the reaction of cyclohexadienes with MMO, but was not found. 2,4-Cyclohexadienol was found to undergo very slow decomposition to form benzene under the experimental conditions, but the rate of the benzene formation by this route is much slower than the observed rate of formation during the MMO-catalyzed oxidation of 1,3-cyclohexadiene. No reaction products were observed when MMOH was omitted.

As in the case of the reaction of ethylbenzene, changes in the reaction conditions alter the distribution among the products substantially (Table 2). H_2O_2-dependent reactions produced more epoxidation than

Table 2 Product distribution from the oxidation of cyclohexadienes catalyzed by MMOH

Substrate reaction system[a,b]	Distribution of products (% of total)[c]		
	Epoxide[d]	Alcohol[e]	Benzene
1,3-Cyclohexadiene			
NADH coupled	32	59	9
Single turnover	29	62	9
H_2O_2 coupled	54	44	2
1,4-Cyclohexadiene			
NADH coupled			
H + 0B + 2R	67	0	33
H + 0.1B + 2R	63	0	37
H + 0.5B + 2R	12	64	24
H + 1B + 2R	10	67	23
H + 2B + 2R	9	65	26
H + 3B + 2R	9	62	29
H + 2B + R	10	64	26
H + 2B + 0.1R	8	64	28
Single turnover			
H + 0B + 2R	66	0	34
H + 2B + 2R	11	58	31
H_2O_2 coupled			
H + 0B	52	15	33
H + 1B	42	18	40
H + 2B	46	16	38

[a]See Materials and methods and the legend to Table 1 for conditions and abbreviations

[b]For NADH-coupled reactions, the reaction mixtures contained MMOH, stoichiometric amounts of MMOB (unless otherwise noted) and MMOR, and an excess amount of NADH. For single-turnover reactions, the reaction mixtures contained MMOH and a stoichiometric amount of MMOB. For H_2O_2-coupled reactions, the reaction mixtures contained MMOH and H_2O_2. Detailed conditions were as described in the Materials and methods section

[c]Relative yields of products determined by comparison with the authentic materials. Equal GC response factors were assumed for the two alcohol products, while it was experimentally found to be true for the two epoxide products. The error for data from single turnover reactions is 10% and for the rest it is 6%

[d]The epoxide products are 3,4-epoxycyclohexadiene and 4,5-epoxycyclohexadiene from the oxidation 1,3-cyclohexadiene and 1,4-cyclohexadiene, respectively

[e]The alcohol products are 2,4-cyclohexadienol and 2,5-cyclohexadienol from the oxidations of 1,3-cyclohexadiene and 1,4-cycohexadiene, respectively

the O_2-dependent reactions that contained stoichiometric MMOB. Decreasing the concentration of MMOB caused more epoxidation for all systems. In contrast, the concentration of MMOR had little effect on the product distribution (Table 2).

Oxidation of other substrates

In additional experiments, trace amounts of desaturated products were detected from the reactions of cumene and cyclohexene. Because of the low yields, these reactions were not further studied. Ethane, ethylene, and cyclohexane were also used to react with MMO, but no corresponding dehydrogenated products were detected with these substrates.

Discussion

The results of this study show that ethylbenzene and cyclohexadienes differ from other substrates for the MMO enzyme system in that desaturated products were generated among other products. This reveals a new desaturase reactivity of MMO in addition to its well-studied hydroxylation and epoxidation activities. The investigation of the reactions of these substrates provides an opportunity to probe the factors that determine the outcome of MMO catalysis. In the following, the relationship between the hydroxylation and desaturation reactions catalyzed by MMO and the implications for the mechanisms of related nonheme diiron proteins are discussed.

Effects of reaction conditions on product distribution

In MMO-catalyzed reactions, ethylbenzene is hydroxylated at the ethyl and phenyl C-H positions and also dehydrogenated at the ethyl group. Similarly, multiple products were generated in the reaction of cyclohexadienes, providing evidence for three types of reactions: hydroxylation, epoxidation, and desaturation. It is important to note that the product distributions of these reactions depend strongly on the reaction conditions and the MMO components present, showing that the reaction chemistry observed is sensitive to the precise physical and/or structural state of enzyme. A good example of this is found in the NADH-coupled reactions with ethylbenzene as the substrate, in which there is preferential hydroxylation on the secondary carbon of the ethyl group in the absence of MMOB, but the addition of MMOB causes a strong bias toward hydroxylation at the *para* position of the benzene ring. A similar preference for the formation of *para*-phenol was also observed in single-turnover reactions with a stoichiometric amount of MMOB present relative to MMOH. In the H_2O_2-coupled reaction, on the other hand, a shift towards hydroxylation at the secondary ethyl C-H and other positions of phenyl group was observed. These observations are consistent with the results of our earlier study on product distribution in which the oxidations of substrates with multiple hydroxylation sites such as isopentane and nitrobenzene were investigated [46]. It was proposed from that study that the observed shifts in regiospecificity of hydroxylation did not result from fundamental changes in the reaction chemistry at the active site, but rather from changes in the way substrates were presented to the active oxygen species. These changes presumably resulted from alterations in MMOH active site structure occurring upon shifts in reaction conditions such as oxidation state of the diiron cluster and component complex formation. The current study shows that, for a select group of substrates, the chemical reaction does, in fact, change as the reaction conditions change. However, just as in the case of shifts in regiospecificity of hydroxylation, the same set of

products emerge from a single type of active site changing only in distribution. Moreover, the effects still occur owing to factors such as protein complex formation which are known to slightly perturb the MMOH active site structure, but seem unlikely to cause large changes in the fundamental oxygen activation chemistry of the diiron cluster [47]. Thus, it is reasonable to propose that the hydroxylation (or epoxidation) and desaturation reactions share a common mechanism up to a critical branch point that is controlled by structural factors, such as the way substrate approaches the activated oxygen species.

The hypothesis that the oxygenation and desaturation reactions of MMO have a closely related mechanism is strongly supported by consideration of two observations. First, all of our transient kinetic studies have shown that substrates react only with compound **Q** [27, 28]. Thus, the oxygen activation steps of the catalytic cycle are likely to be the same for hydroxylation and desaturation reactions. Second, the novel desaturase activity of MMO does not apply to all substrates, notably saturated hydrocarbons. In the cases reported here, the substrates that were desaturated all contain a suitably juxtaposed conjugated system to the targeted C-H bond. Together these observations suggest that the difference in reactivity occurs during the reaction of **Q** with the substrate and the outcome depends on the nature of the substrate rather than novel chemistry of MMO.

Proposed mechanism for hydroxylation and desaturation

The chemical mechanism of MMO catalysis has been extensively studied in recent years, but remains controversial [1, 2]. It is our contention, based on various mechanistic studies, that the hydroxylation reaction of methane proceeds in stepwise fashion and involves a hydrogen abstraction process [5, 49]. We have also proposed the involvement of radical and/or cation intermediates for a variety of other substrates [41, 50, 51].

The mechanism we proposed previously for the formation of the usual alcohol product of MMO catalysis (Fig. 1) also contains the elements necessary to rationalize the formation of the desaturated products, as illustrated in Fig. 3. The products from both hydroxylation and desaturation of a substrate like ethylbenzene may be envisaged to arise by a common process in which the first step is the abstraction of a hydrogen atom to form a radical. The radical may either generate the alcohol by capturing the iron-bound hydroxyl or lose another electron to become a carbocation. The latter process is possible because, after the initial hydrogen abstraction step, the metal center is formally in the mixed-valent Fe(III)Fe(IV) state, which is equivalent to the oxidation state of the intermediate **X** of the RNR-R2 reaction cycle [32]. At this stage, the cluster is still electron deficient and capable of electrophilic attack, as

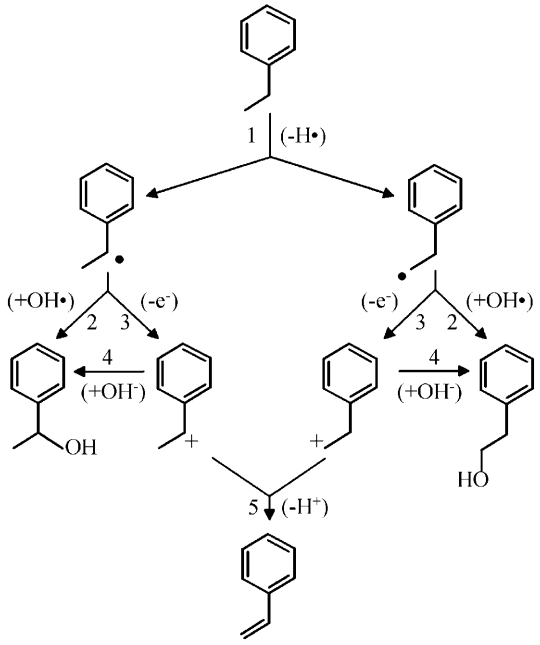

Fig. 3 A proposed scheme for the hydroxylation and desaturation of ethylbenzene catalyzed by MMO. (*1*) Hydrogen abstraction; (*2*) capture of the radical to form alcohol; (*3*) electron abstraction; (*4*) capture of the cation to form alcohol; (*5*) proton abstraction

demonstrated by the function of **X** in generating an endogenous tyrosyl radical. Also, MMO-like reactivity has been observed for a synthetic high-valent Fe(III)-Fe(IV) complex [52]. Like the substrate radical species in our proposed mechanism, the cation resulting from the abstraction of a second electron could be captured by the hydroxide group to give the alcohol, but it could also generate the desaturated product by the loss of a proton (Fig. 3, step 5). Although this mechanism is attractive based on our previous studies, generation of a substrate cationic intermediate by other mechanisms, such as direct hydride transfer to compound **Q**, cannot be ruled out based on available data[3].

In our view, the movement of the electrons and protons in the mechanism shown is closely correlated, and the precise timing could be influenced by the nature of the substrate. Substrates with low ionization potential would have a stronger tendency for the second electron abstraction process (Fig. 3, step 3) in competition with step 2, whereas the propensity of the cation to lose a proton would determine the competition between steps 4 and 5 and ultimately the amount of desaturated product formed. A conjugated system in the substrate would provide strong stabilizing energy to the intermediate, be it a radical or a cation, which would also lead to longer

[3]Another means to form a cation would be to invoke intermediate **P** of the reaction cycle, which is likely to contain a protonated peroxy species [53]. Direct attack of this species on a substrate might yield a cationic intermediate [54]. However, intermediate **P** has thus far not been observed to react with any substrate in transient kinetic studies of the *M. trichosporium* OB3b MMOH reaction cycle

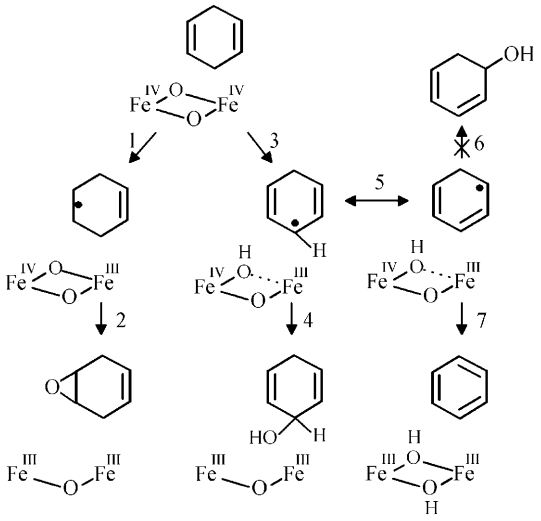

Fig. 4 A proposed scheme for product formation from the oxidation of 1,4-cyclohexadiene catalyzed by MMO. (*1*) Electron abstraction; (*2*) oxygen radical rebound and epoxide formation; (*3*) hydrogen abstraction; (*4*) hydroxylation; (*5*) radical resonance; (*6*) blocked hydroxylation step; (*7*) electron abstraction and elimination

lifetimes for these intermediates and a better chance for the elimination reaction to occur.

In the case of the cyclohexadienes, a similar mechanism can be written to account for the products except that electron abstraction from a double bond leading to epoxide formation must also be postulated (Fig. 4, steps 1 and 2). Hydrogen atom abstraction would again be the first step in both the hydroxylation and desaturation reactions (step 3). The fact that no rearrangement of the double bond structure of 1,4- and 1,3-cyclohexadienes occurs during formation of the respective alcohol products suggests that the substrates do not change their orientation in the active site relative to the reactive oxygen species during the reaction. Thus, the hydroxylation reaction would involve simple rebound to the position of initial abstraction (step 4). On the other hand, resonance stabilization of the radical would occur (step 5) and result in a decrease in the radical character of this position in favor of a position too far distant from the reactive oxygen species for rebound (step 6). This net increase in electron density at the carbon where the initial hydrogen atom abstraction occurred would promote the competing second electron abstraction reaction to yield the desaturation product, benzene (step 7).

It is interesting to note that, in the reconstituted NADH-coupled MMO system with stoichiometric MMOB, the relative amounts of epoxide and desaturated products are approximately interchanged for the reactions of the 1,3- and 1,4-cyclohexadiene substrates. This may arise simply from the way in which these substrates are bound in the active site due to the fact that the ring conformations will be quite different. On the other hand, in accord with the mechanistic proposal described here, the *cis* double bond structure of 1,3-cyclohexadiene would provide better stabilization of a radical species resulting from electron abstraction and consequently enhance epoxide formation. Similarly, following hydrogen atom abstraction, electron density will be shifted somewhat in favor of the basis resonance structure with *cis* double bonds, promoting hydroxylation in the case of 1,3-cyclohexadiene and desaturation for 1,4-cyclohexadiene, as observed.

Based on the general model proposed here, the competition between the formation of the C-O bond and the loss of the equivalent of another hydrogen atom from the substrate would be strongly affected by the positioning of the substrate relative to the activated oxygen at the iron center. That, perhaps, is the reason for the different ratios of alkene to alcohol for a single substrate under different enzyme conditions that induce conformational changes of the enzyme. Similarly, the low yield of dehydrogenated product from cumene could stem from the large steric hindrance at the α-carbon of cumene (tertiary carbon) compared to that in ethylbenzene (secondary carbon). The slower reaction with cumene catalyzed by the enzyme might cause the lower rate of formation of all products, including both alcohol and styrene. In contrast, the lower amount of desaturation in the case of cyclohexene may arise for a different reason. The stabilizing force from one double bond is, perhaps, not enough to significantly change the rates of the reaction; consequently, little leakage of the oxidizing power in the direction of desaturation occurs.

A close mechanistic kinship between oxygenation and desaturation reactions has also been found in several other systems, including cytochrome P450 monooxygenase [55], naphthalene dioxygenase [56], and bleomycin [57]. The natural function of these systems is oxygenation, but they all exhibit desaturation activities with certain substrates.

Implications for the chemical mechanisms of related enzymes

Although the ability to catalyze desaturation reactions is a novel function for MMO, it is not so for nonheme diiron clusters as a group because it is the primary reaction for Δ9D [12, 36, 37]. The observation of desaturase catalysis by MMO is consistent with the proposal that similar chemical processes occur in the structurally related diiron cluster-containing enzymes. Our results suggest that the difference in their reaction outcomes may stem from subtle changes in the active site cluster environment, as demonstrated here for MMO. A similar conclusion was reached from studies of Δ9D in which it was shown that mutagenesis of only a few key residues resulted in a switch from desaturation to hydroxylation, just the opposite shift in the primary reaction chemistry as observed here for MMO [58]. Similarly, isotope effect studies of the reaction of *Arabidopsis thaliana* Δ^{12}-desaturase supported the hypothesis that hydrogen atom abstraction yields a substrate

radical intermediate which can proceed to the desaturated product by either simple disproportionation or a two-step oxidation/deprotonation, as we propose here [59]. It is important to note that the desaturase enzymes yield dehydrogenated products without special stabilization of the position that is attacked in the substrate as required in the case of MMO. Thus, the protein environment plays a critical role in determining whether desaturation or hydroxylation is favored.

Recently, the ability of a single diiron cluster to catalyze the types of reaction discussed here was demonstrated through the use of the synthetic dinuclear iron complex $[Fe(III)Fe(IV)(\mu\text{-}O)_2(TPA)_2]^{3+}$ {TPA = tris (2-pyridylmethyl)amine} [52]. This complex carries out a range of oxidation reactions corresponding to those associated with MMO, Δ9D, and RNR-R2, including hydroxylation and desaturation.

In summary, with substrates such as ethylbenzene and cyclohexadienes, MMO catalyzes not only the conventional hydroxylation reaction and/or epoxidation reactions, but also the desaturation reaction. This new activity of MMO is postulated to be associated with the chemical properties of the substrates rather than fundamental changes in the nature of the oxygen activation and C-H activation chemistry. The results also suggest that the properties of the substrates and structural parameters of the active site play central roles in determining the outcome of MMO reaction.

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