A Concerted Mechanism for Ethane Hydroxylation by the Particulate Methane Monooxygenase from *Methylococcus capsulatus* (Bath)

Barrie Wilkinson,[†] Mei Zhu,[‡] Nigel D. Priestley,[†,⊥] H.-Hoa T. Nguyen,[‡] Hiromi Morimoto,[§] Philip G. Williams,[§] Sunney I. Chan,*[,‡] and Heinz G. Floss*[,†]

Department of Chemistry, Box 351700, University of Washington, Seattle, Washington 98195-1700
A. A. Noyes Laboratory of Chemical Physics
California Institute of Technology
Pasadena, California 91125
National Tritium Labeling Facility
Lawrence Berkeley National Laboratory 75-123
1 Cyclotron Road, Berkeley, California 94720

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Methane monooxygenase (MMO) catalyzes the oxidation of methane to methanol in methanotrophic bacteria.[1] The study of such C−H bond activation is an area of significant current interest.[2]

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

Two distinct forms of MMO exist, a non-heme iron-containing soluble cytosolic form (sMMO)[2] and a copper-containing particulate form (pMMO), which is membrane bound.[3,4] Under growth-limiting conditions, pMMO is the major monooxygenase expressed in methanotrophs that produce both particulate and soluble forms of the enzyme.[5] Indeed, when the concentration of copper ions is sufficient, expression of the sMMO is suppressed. Recent studies have demonstrated that pMMO has an obligate requirement for copper ions[4] and that, in isolated membrane fractions containing pMMO activity, the enzyme activity increases with increasing copper concentration. Further evidence has been obtained suggesting that copper added to such membrane fractions is incorporated into the putative enzyme active site.[6] Until recently, investigations of the pMMO mechanism have been limited due to the problems of handling an enzyme that proves difficult to isolate.[6c] The mechanism of C−H bond activation, however, has been extensively studied for sMMO isolated from both *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath).[2] These studies, many involving "radical probes",[7] have led to mechanistic proposals invoking alkyl free radical or carbocation intermediates[2,8,9] or concerted oxygen insertion into a C−Fe bond.[10]

We have recently studied the mechanism of alkane hydroxylation by sMMO from *M. trichosporium* OB3b using as a probe ethane bearing a chiral methyl group.[11] The conversion of (*S*)- or (*R*)-[1-^{3}H$_1$,^{2}H$_1$]ethane into ethanol was found to proceed with predominant (68%) retention, accompanied by significant (32%) inversion of configuration.[11−13] Similar data were obtained with the sMMO from *M. capsulatus* (Bath).[14] These results point to a concerted, nonsynchronous mechanism involving an extremely short-lived alkyl radical structure in the transition state, which can undergo partial rotation about the C−C bond. A similar mechanism has recently been proposed for rat liver P-450 catalyzed hydroxylations.[15] Based on the barrier to C−C bond rotation in ethyl radicals[16] (0.15 kcal mol^{-1}), one calculates the rate for this rotation to be 4.9×10^{12} s^{-1} at 30 °C. Based on this rate, we can obtain, to a first approximation, a rate constant of 5.5×10^{12} s^{-1} for the postulated "radical capture" step of this reaction sequence,[12,14] i.e., a lifetime of about 180 fs for the radical structure.

The same approach[11] was used in the present study of the cryptic stereospecificity of alkane hydroxylation by pMMO. Reaction of carrier-free LiEt$_3$B^{3}H[17] with (*S*)- or (*R*)-[1-^{2}H$_1$]- and (*S*)- or (*R*)-[1-^{2}H$_1$, 2-^{2}H$_3$]ethyl tosylates (>98 atom % ^{2}H, 88 ± 4% ee) gave the corresponding ethane isotopomers bearing a chiral methyl group. These substrates were then incubated with pMMO-containing membranes, NADH, and O$_2$.[18] The reaction mixtures were lyophilized to recover alcohol/water mixtures as products (yields, 68−153 mCi), which were analyzed by ^{3}H NMR. This analysis allowed calculation of a primary intramolecular kinetic isotope effect (KIE) of k_H/k_D = 5.2 ± 0.4 for the [1-^{3}H$_1$,^{2}H$_1$]ethane experiments and k_H/k_D = 5.5 ± 0.7 for the [1-^{3}H$_1$,^{2}H$_1$, 2-^{2}H$_3$]ethane experiments. Analysis of the total ^{3}H distribution showed an average of 83% tritium in the [1-^{3}H$_1$,^{2}H$_1$]ethane and 73% in the [1-^{3}H$_1$,^{2}H$_1$, 2-^{2}H$_3$]ethane incubation products present as H^{3}HO. This value is much greater than the average 10%−15% H^{3}HO found in experiments with sMMO[11,12,14] and far greater than the theoretical maximum value based on even a KIE = 1.[19] The possibility of further oxidation of the product to H^{3}HO and CO_2 was examined by incubation of the enzyme preparation with [1-^{14}C]ethanol. This produced no appreciable $^{14}CO_2$.[20] It is

* Author to whom correspondence should be addressed.
[†] University of Washington.
[‡] California Institute of Technology.
[§] Lawrence Berkeley National Laboratory.
[⊥] Present address: College of Pharmacy, The Ohio State University, Lloyd M. Parks Hall, 500 West 12th Ave., Columbus, OH 43210-1291.

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(18) Incubations were performed for 12−15 min at 45 °C in the presence of formaldehyde (5 μM) in 50 mM PIPES buffer, pH 7.1, total volume of 2.3 mL. Incubation with each isotopomer was performed in duplicate.
(19) Calculation of the tritium distribution in the methyl/methylene/water positions based on unity and infinite isotope effects gives the range 3:2:1 to 3:1:0.
(20) To test for further oxidation of ethanol by pMMO, [1-^{14}C]ethanol (8 μCi) was incubated as substrate under conditions identical to those for the ethane incubations. The head space gases were isolated and stirred over NaOH. After workup, the $Na_2{}^{14}CO_3$was quantified by scintillation counting (0.04 μCi, 0.5% overall conversion). Incubation was performed in duplicate.

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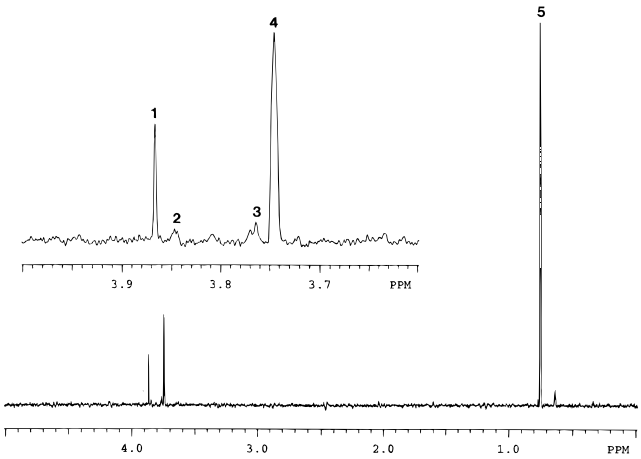
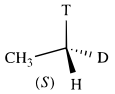
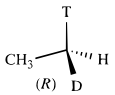
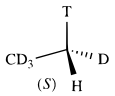
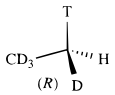


Figure 1. ^{3}H NMR spectrum (640.13 MHz, ^{1}H decoupled) of the (2*R*)-2-acetoxy-2-phenylethanoate derivative of ethanol obtained from the incubation of (*R*)-[1-^{2}H$_1$,^{3}H$_1$]ethane with pMMO from *M. capsulatus* (Bath): (1) (*R*)-[1-^{3}H$_1$]ethanol; (2) (*R*)-[1-^{2}H$_1$,^{3}H$_1$]ethanol; (3) (*S*)-[1-^{3}H$_1$]ethanol; (4) (*S*)-[1-^{2}H$_1$,^{3}H$_1$]ethanol; (5) [2-^{2}H$_1$,^{3}H$_1$]ethanol. NMR signal assignments were made according to the method of Parker.[25]

clear from experiments with formaldehyde as an inhibitor,[21] however, that further modification of the product ethanol does occur (not too surprising in view of the "crude" and complex nature of the enzyme preparation presently available), although its nature remains obscure. Oxidation of a sample of ethanol from a hydroxylation of chiral ethane to acetate, followed by configurational analysis of the chiral methyl group,[22] demonstrated that no racemization, and therefore no washout of isotopes, occurs at C-2, either during synthesis or incubation.[23] Washout at C-1 due to an oxidation/reduction cycle catalyzed by a contaminating alcohol dehydrogenase also is not very likely, as this would lead to a distortion of the apparent stereochemical results,[14] which is not observed. One possibility that could account for the observed formation of H^{3}HO is oxidation of an alkoxy species (which cannot be formed by binding of ethanol to the enzyme) to CO_2 and H^{3}HO prior to its release from the enzyme.

The ethanol samples in the isolated alcohol/water mixtures were converted into their (2*R*)-2-acetoxy-2-phenylethanoate derivatives[25] (2−34 mCi). Examination of the well-resolved ^{3}H NMR spectra (Figure 1) for these derivatives produced an exceptionally consistent set of stereochemical data (Table 1). When corrected for the enantiomeric purity of the ethyl tosylate starting materials, the data clearly show that the reaction occurs with complete retention of configuration, i.e., with 100% stereoselection.[26] Barring substantial slowing of the carbon−carbon bond rotation of the ethyl radical when bound to the enzyme,[27] this result rules out mechanisms proceeding via alkyl radical (and/or cation) structures, even very short-lived ones, as such intermediates would have to have a lifetime of $<1 \times 10^{-14}$ s in order not to undergo any detectable C−C bond rotation, i.e., the capture reaction would have to be much faster than the decay of a transition state.[28] The data instead point to a mechanism in which C−H bond cleavage is preceded by bond formation at the alkyl carbon, i.e., one proceeding through a pentacoordinated carbon species. Whether this initially formed bond is a C−O or a C−metal bond remains to be established.

Table 1. Percentage Distribution of ^{3}H Label in the Methylene Group of the (2*R*)-2-Acetoxy-2-phenylethanoate Derivatives of the Ethanol Samples Generated from Chiral Ethane by *M. capsulatus* (Bath) Membranes Containing pMMO

Substrate	Product #4	Product #1	Product #2	Product #3
	8[b]	2[b]	75[b]	15[b]
	Retention = 97%[a,c]			
	77[b]	17[b]	4[b]	2[b]
	Retention = 100%[a,c]			
	2[b]	3[b]	75[b]	20[b]
	Retention = 100%[a,c]			
	80[b]	14[b]	2[b]	4[b]
	Retention = 101%[a,c]			

[a] Overall retention data have been corrected for the enantiomeric purity of substrates. [b] These data were obtained directly from spectra. [c] The deviation from 100% is well within the compounded error range of NMR integration for product and substrate spectra. The numbering scheme shown here for the products corresponds with the peak numbering in Figure 1.

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(23) An aliquot of the alcohol/water product from an incubation with (*S*)-[1-^{2}H$_1$,^{3}H$_1$]ethane was oxidized with aqueous $KMnO_4/K_2CO_3$ to (*S*)-[2-^{2}H$_1$,^{3}H$_1$]acetate,[12,14] the enantiomeric purity of which was analyzed by a procedure used routinely in our laboratory.[22c] The configurational analysis gave an *F* value[24] of 26, corresponding to 82% ee *S* configuration, in close agreement with the optical purity of the starting material (88% ee).

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(26) This finding cannot be explained by any kinetic resolution due to the observed further oxidation of an intermediate alkoxy species, since both enantiomers of the substrate give the same result.

(27) Such a phenomenon, considered very unlikely, was excluded in the studies with both sMMOs but could not be probed with the pMMO.

(28) When $\Delta G^{\#} = 0$, (i.e., the activation free energy for decomposition of a transition state), the rate constant at 45 °C is 6.6×10^{12} s^{-1}.[29]

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