Selective Hydroxylation of Small Alkanes by the Particulate Methane Monooxygenase

Thesis by

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Acknowledgment

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Abstract

The particulate methane monooxygenase (pMMO) catalyzes the oxidation of methane to methanol under ambient temperatures and pressures. Other small alkanes and alkenes are also substrates of this enzyme. We measured and compared the initial rate constants of oxidation of small alkanes (C1 to C5) catalyzed by pMMO. Both primary and secondary alcohols were formed from oxidation of n-butane and n-pentane. The alcohols produced from alkane oxidation can be further oxidized, probably by pMMO, to aldehydes and ketones. The apparent regioselectivity for n-butane and n-pentane is 100% 2-alcohols because the formation of primary alcohols is slower than further oxidation of these alcohols. The hydroxylation at the secondary carbons is highly stereoselective: (R)-alcohols are preferentially formed. The enantiomeric excess increases slightly with decreasing reaction temperature. The steric course of hydroxylation on primary carbons was also studied by using isotopically substituted ethane: (S)- or (R)-CH_3-CHDT, and (S)- or (R)-CD_3-CHDT and the reactions were found to proceed with 100% retention of configuration. A primary isotopic effect of k_H/k_D=5.0 was observed in these experiments.

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Introduction

The selective activation of carbon-hydrogen bonds (C-H) in unfunctionalized alkanes has been a challenge to the industrial and academic chemists for many years. The C-H bonds in alkanes are relatively strong and nonpolar, which leads to a high kinetic stability. The most common reactions of alkanes are oxidation and re-forming processes typically involving radical chain and carbonium ion intermediates, which are prone to low selectivity in the product distribution. In contrast, many living systems contain enzymes that oxidize alkanes at ambient temperatures and low oxygen pressures. Examples are cytochrome P-450, a-hydroxylases, and methane monooxygenase (MMO).

MMO catalyzes the NADH and O_2-dependent oxidation of methane as well as other alkanes to the corresponding alcohols:

$$CH_4 + NADH + O_2 + H^+ \longrightarrow CH_3OH + NAD^+ + H_2O$$

Aside from the hydroxylation of alkanes and alkenes, MMO can also catalyze the epoxidation of internal and terminal alkenes. Two distinct MMO enzymes have been observed which differ in cellular location: a soluble methane monooxygenase (sMMO) expressed under limiting copper growth conditions and a membrane-bound form (pMMO) expressed during growth in the presence of excess copper. To date, the sMMO from four strains of bacteria has been isolated;[1] and the crystal structure of the dimeric hydroxylase from *Methylococcus capsulatus* (Bath) has been solved.[2] Several laboratories have also studied the mechanism of hydroxylation mediated by the sMMO.[3-6] Whether the hydroxylation proceeds through a substrate radical intermediate, or a concerted pathway is involved in the C-H bond oxidation insertion, is still an issue of controversy. Considering the wide range of substrates that the enzyme can utilize, the sMMO may operate by more than one mechanism. Thus, the mechanistic pathways might be substrate-dependent. In contrast, the substrate specificity of pMMO is quite narrow. It catalyzes the oxidation of

only small hydrocarbons containing 1 to 5 carbons. Little is known about the structure and mechanism of the particulate enzyme despite its diverse presence in methanotrophs. Trinuclear copper clusters have recently been proposed as the active site for this enzyme.[7,8]

The substrate specificities of the sMMO and pMMO have been studied and the types of reactions catalyzed by these enzymes are known.[9-12] However, there is only limited information regarding the regio- and stereo-selectivity of these reactions. The sMMO does not exhibit high product selectivity in most alkane hydroxylation reactions. Various mixtures of primary, secondary, or even tertiary alcohols have been observed in the final hydroxylation products. In contrast, the pMMO appears to be more regiospecific than the sMMO. In any case, the stereospecificity of the reactions catalyzed by these two enzymes has not been investigated to any appreciable extent. Hydroxylation of chiral ethane catalyzed by the sMMO from *Methylosinus trichosporium* OB3b revealed only partial retention (~65%) of configuration in the isotopically substituted ethanol products. The stereospecificity of the hydroxylation reactions catalyzed by pMMO has never been addressed in the literature. The work presented in this report is directed toward gathering these crucial data which could provide significant insight into the mechanistic pathway(s) of the pMMO catalysis.

In studies to determine the regioselectivity of alkane oxidation catalyzed by pMMO, we observed further oxidation of both primary and secondary alcohols. Although such phenomena were reported by other researchers in the late 1970's [13-15], it was thought that oxidation of alcohols was due to methanol dehydrogenase or other nonspecific alcohol dehydrogenases present in the whole cells or cell free soluble crude extracts. However, our experimental results indicate that this might be not true in the case of the pMMO system. Particulate MMO may actually be capable of further oxidizing alcohols to aldehydes and ketones.

Materials and Methods

A. Growth of Methanotroph and Preparation of Membrane Extracts

Methylococcus capsulatus (Bath) cells were grown and harvested under conditions as previously reported, except that copper sulfate concentration was raised to 50 μM.[7] Particulate MMO was expressed exclusively under these conditions. Membrane extracts were also prepared as previously described.[7] Membrane extracts were washed three times to remove soluble enzymes and small molecules before they were frozen at liquid nitrogen temperature dropwise. Frozen membranes were stored at -80 C until used.

B. MMO Activity Assay and Rate Constant Measurement of Alkane Oxidation

The pMMO activity was measured by the standard propene epoxidation assay.[16] Alkane oxidations catalyzed by pMMO were carried out at desired temperatures in the following manner. Solutions were prepared that contained 0.1 g of frozen membrane extracts, 5 mg of NADH, and 1mL of 20 mM PIPES (pH 7.1) in a 10 mL sealed serum vial. After evacuating the head space air, the serum vial was placed in a water bath maintained at the desired temperature. The reaction was then initiated by injecting 4 mL each of oxygen and substrate gases (Mathesons, 99% purity) into the vial. The reaction mixture was vigorously stirred with a magnetic stir bar.

Products from enzyme oxidations were analyzed with a Hewlett-Packard (HP) Model 5840 gas chromatography with an FID detector using a preparative column (AT-1000, supported on cross-linked carbograph, Alltech). Products were identified and quantified by comparing the retention times and the area integral with the authentic standards at known concentrations.

To measure the initial rate constants of alkane oxidations catalyzed by pMMO, 1 or 2 μL of reaction mixture was withdrawn from the sealed serum vial and injected into the GC at different reaction time, and reaction progress curve was recorded. In cases when the sampling time had to be shorter than the time needed to finish analyzing the sample on GC

with desired resolution, 10~15 μL of reaction mixture was withdrawn from the sealed reaction vial at selected period of time, transferred into eppendorf microtubes, and analyzed later. The microtubes were placed in ice to quench the reaction before the samples were analyzed by GC. Initial rate constants were obtained by deviding the initial slope of the progress curves by the total amount of protein in the sample determined by the Lowry Method.[21]

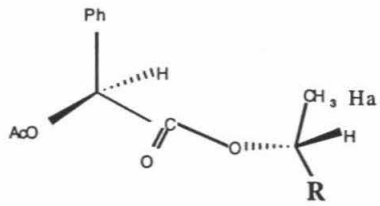
In studies of alcohol oxidation, alcohols were used directly as the substrates, and the initial rate constants were measured in a similar way as the alkane oxidation constant.

C. Study of Stereospecificity of Secondary Alcohols from Alkane Oxidation

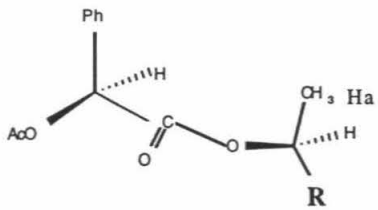
Hydroxylation at the secondary carbon positions of n-butane and n-pentane generates chiral carbon centers in these two molecules. This enables us to study the enantioselectivity of these reactions by determining the enantiomer ratios in the products.

Either whole cells or membrane extracts were employed as the pMMO source to catalyze the oxidation of n-butane and n-pentane. Sodium formate was used as the electron donor in the whole cell system. Reactions incubated with the whole cells were allowed to proceed for a few hours, while only ~10 minutes was needed to achieve the same amount of alcohol products in reactions incubated with the membrane extracts. The reactions were terminated by placing the reaction flask into the ice, the cells or the membrane extracts were removed from the reaction mixture by centrifugation. The alcohols were extracted three times with ethyl ether and concentrated by carefully evaporating the ether at low temperature (10 °C).

For determination of their absolute configurations, the alcohols were converted into diastereoisomers by (S)-2-acetoxy-2-phenylethanoic acid at -10 °C.[17] The (S,R)[18] and (S,S) ester derivatives of these alcohols are designated below as A and B, respectively.



A (S,R)



B (S,S)

R_1= — CH_2-CH_3
R_2= —CH_2-CH_2-CH_3

^{1}H-NMR spectra (see Fig. 1) of the ester compounds at 300 MHz reveal that the methyl protons (H_a) in the (S,S) diastereoisomer resonate at a higher field than those in the (S,R) diastereoisomer. The areas obtained upon integration of these well-resolved doublets enabled us to quantify the R/S ratio of the alcohol products formed from the pMMO-catalyzed hydroxylation. The diastereoisomer product mixtures were also analyzed independently by HPLC using a D-DNB-Leucine chiral column.[19] Results obtained by this method are consistent with NMR analysis.

D. Study of Stereospecificity of Primary Alcohols from Alkane Oxidation

To follow the steric course of primary alcohol formation, isotopically substituted ethanes: (S)- or (R)-CH_3-CHDT, (S)-or (R)-CD_3-CHDT, were used as the substrates for pMMO catalyzed alkane oxidation. These experiments were carried out at the Lawrence Berkeley Laboratory, in collaboration with the people in the National Tritium Laboratory.

The ethane samples were synthesized by reaction of carrier-free $LiEt_3BT$ with the known (R)- or (S)-[1-D]ethyl tosylate (>98 atom % D, 88% ee) and incubated with the membrane extracts. A precise amount of formaldehyde (varying from 6 to 30 mM from run to run) was added into the reaction flask to prevent the further oxidation of ethanol. The incubation proceeded for a short time (varying from 8 to 30 minutes from run to run) before it was quenched at liquid nitrogen temperature. Following the addition of unlabeled carrier ethanol, each sample was then lyophilized, and the recovered liquid was analyzed by

^{3}H-NMR. This analysis revealed the percentage of tritium released into water and the ratio of oxidation at the labeled versus the unlabled methyl group. Each ethanol sample was then derivatized to the (R)-2-acetoxy-2-phenylethanoate ester, which was again analyzed by ^{3}H-NMR spectroscopy. The resonances of the four diastereoisomers of the derivatives are well enough resolved to allow accurate quantitation.

Results and Discussion

I. Regioselectivity of alkane oxidation catalyzed by pMMO

(A). Formation of primary alcohols

Primary alcohols are formed from the oxidation of small alkanes catalyzed by pMMO. The whole process can be described by the following equation:

$$R\text{-}CH_3 + O_2 + NADH + H^+ \xrightarrow[k_A]{pMMO} R\text{-}CH_2\text{-}OH + NAD^+ + H_2O$$

$$R = H \longrightarrow C_5$$

The progress curve of methane and ethane oxidation are shown in Fig. 2 and Fig 3. The reaction rate k_A, which is tabulated in Table I, differs significantly from small substrates, such as CH_4 and C_2H_6, to relatively large substrates, such as butane and pentane.

(B). Formation of secondary alcohols

Secondary alcohols were also formed upon oxidation of n-butane and n-pentane in the following reaction:

$$R\text{-}CH_2\text{-}CH_3 + O_2 + NADH + H^+ \xrightarrow[k_B]{pMMO} R\text{-}CH(CH_3)\text{-}OH + NAD^+ + H_2O$$

$$R = -C_2H_5, \text{ or } -C_3H_7$$

Fig. 4 and Fig. 5 are the progress curves for these two reactions. The rate constant for these two reactions, k_B are also tabulated in Table I. It is interesting to note that the rate

constants for primary alcohol formation (k_A) is on the same order for butane and pentane, while their k_B's differ by almost one order.

Table I. Rate constant for primary and secondary alcohol formation at 45 °C.

Initial rate constant [μM/(min mg protein)]	substrate			
	CH_4	C_2H_6	n-C_4H_{10}	n-C_5H_{12}
k_A	86.7	102.1	3.1	2.2**
k_B			29.4	5.5*

* This was the fastest rate observed

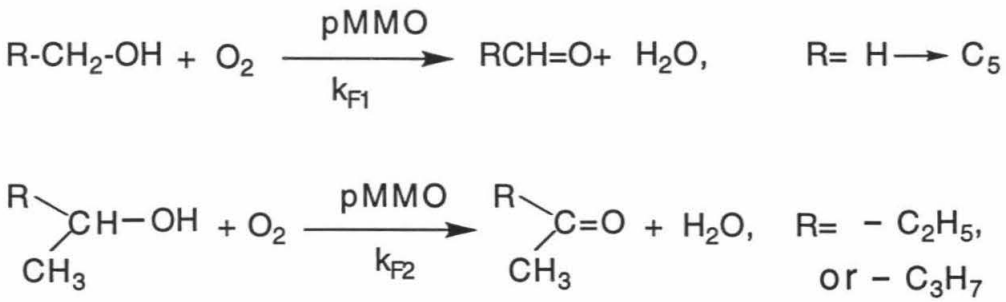
** $k_A = k_B$ * (1-alcohol/2-alcohol) $_{inhibitor}$. See discussion in section C. and D.

(C). Further oxidation of primary and secondary alcohols by pMMO

Further oxidation of alcohols was considered to be catalyzed by methanol dehydrogenase (MDH) or other nonspecific alcohol dehydrogenases in early research using the whole cells or the soluble crude extracts as the MMO source. MDH is a soluble periplasmic quino protein present in gram negative bacteria grown on methane or methanol. We therefore expected much less MDH present in our carefully washed membrane extracts than in the whole cells. However, we observed a much faster ethanol oxidation rate with the membrane than with the whole cells. Furthermore, MDH requires ammonium salt as an activator and primary electron acceptor (such as phenazine ethosulfate PES) for its activity and has its optimal activity at pH=9. However, our membrane extracts readily oxidize ethanol in the absence of ammonium salt and presumed electron acceptor. The optimal activity was found at pH~7.1. Formaldehyde was found to inhibit the further oxidation of the alcohols (compare Fig. 2 and Fig. 6); it also slowed down the overall alkane oxidation as well. Three progress curves of ethane oxidation to ethanol in the presence of different amounts of formaldehyde are shown in Fig. 7. It is obvious that the reaction with the least amount of formaldehyde has the fastest rate constant. This indicates that alkane

hydroxylation and further alcohol oxidation might be catalyzed by the same enzyme: pMMO.

Both primary and secondary alcohols can be oxidized irreversibly to aldehyde and ketones by membrane extracts.



The rate constants for these reactions depend on the size of the substrate and the position of the hydroxyl group. k_{F1} is ten times larger than k_{F2}, and their values are tabulated in Table II.

Table II. Rate constant for oxidation of alcohols catalyzed by pMMO at 45 °C.

Initial rate constant [μM/(min mg protein)]	substrate			
	methanol	ethanol	butanol	pentanol
k_{F1}		19.6	3.2	2.5
k_{F2}			0.4	0.2

Detailed studies on the oxidation of alcohols have been carried out in order to have a better understanding of this process. The rate constants of ethanol oxidation were measured at different temperatures, pH, oxygen concentration, and in the presence of formaldehyde inhibitor and these values were tabulated in Table III. Artificial electron acceptor (NAD^+) slightly retards this reaction while electron donors (NADH) enhances this reaction by increasing the initial reaction rate as well as the time that the enzyme remains

active. The extent of enhancement by NADH depends on the substrate specificity: its effect on oxidation of primary alcohols is much more significant than on secondary alcohols. Fig. 8 describes the oxidation of 1-butanol under three different conditions: small amount (~0.3 mM) of butyraldehyde is enough to inhibit completely the further oxidation of 1-butanol; however, in the presence of NADH (3 mM), the enzyme seemed to be reactivated, and oxidation of 1-butanol by pMMO proceeded for a longer time (> 60 min) until the butyraldehyde concentration reaches ~1.5 mM. In another words, pMMO can tolerate much higher level's of butyraldehyde in the presence of coenzyme NADH.

The electrons from the alcohol oxidation probably flow from the alcohol to the dioxygen, presumably through the electron transfer chain. This is indicated by the significant decrease in rate constant of ethanol oxidation when the head space air of the reaction vial was removed.

Table III. The rate constants for further oxidation of ethanol catalyzed by pMMO at different reaction conditions. Reactions were carried out at 45 °C and pH=7.1 unless otherwise noted.

reaction condition	pH=7.1	pH=9.3	T=25 °C	*O_2 limiting	HCHO (7 mM)
k_{F1}(μM/(min mg protein)	19.6	2.0	1.0	3.5	0

* The head space air of the reaction vial was removed.

D. Regioselectivity

From section A and B, we have learned that the rate constant for the formation of secondary alcohol (k_A) is ten times larger than the formation of the primary alcohol (k_B), for substrates butane and pentane. The opposite trend in rate constant was observed in the further oxidation of the corresponding alcohols: k_{F1} is ten times larger than k_{F2}. Furthermore, the rate constants of the formation of 1-butanol and 1-pentanol are actually smaller than the rate constants of their further oxidation. So secondary alcohols were the

only alcohol products observed in the oxidation of n-butane and n-pentane catalyzed by pMMO. The apparent regioselectivity is as following.

$$\text{Regioselectivity} \sim \left(\frac{\text{2-alcohol}}{\text{1-alcohol}} \right)_{\text{apparent}} > 100/1$$

The real regioselectivity, measured when the further oxidation of alcohols was inhibited by the addition of formaldehyde, was much less than 100/1.

$$\text{Regioselectivity} \sim \left(\frac{\text{2-butanol}}{\text{1-butanol}} \right)_{\text{HCHO inhibitor}} \sim 10/1$$

$$\text{Regioselectivity} \sim \left(\frac{\text{2-pentanol}}{\text{1-pentanol}} \right)_{\text{HCHO inhibitor}} \sim 2.5/1$$

II. stereoselectivity of the secondary alcohols

The enantiomer ratios of 2-butanol and 2-pentanol from the oxidation of n-butane and n-pentane at three different temperatures have been determined, and their values are listed in Table IV, together with the calculated enantiomeric excesses [ee = (R-S)/(R+S)] for the two substrates under consideration. Figure 9 is a typical ^{1}H NMR spectrum of the (R)-2-acetoxy-2-phenylethate derivative of 2-butanol generated by butane oxidation catalyzed by pMMO. The stereoselectivity of the reactions incubated with the whole cells agrees very well with that of the reactions incubated with the membrane extracts despite their orders of magnitude difference in incubation time (see Fig. 10). This suggests that side reactions or further metabolism in the whole cells do not mask the stereoselectivity of alkane oxidation. Both enantiomers were formed for both substrates in the pMMO-catalyzed hydroxylation. However, a surprisingly strong bias towards the (R)-2-alcohols was observed in the

products. This bias is somewhat affected by the reaction temperature. The lower the reaction temperature, the higher the enantioselectivity. The size of the substrate also has a significant effect on the chiral distribution of the products. We observed a large enantioselectivity increase from n-butane to n-pentane.

Table IV: Distribution of absolute configuration and enantiomeric excesses of products obtained from pMMO-catalyzed hydroxylation of n-butane, and n-pentane.

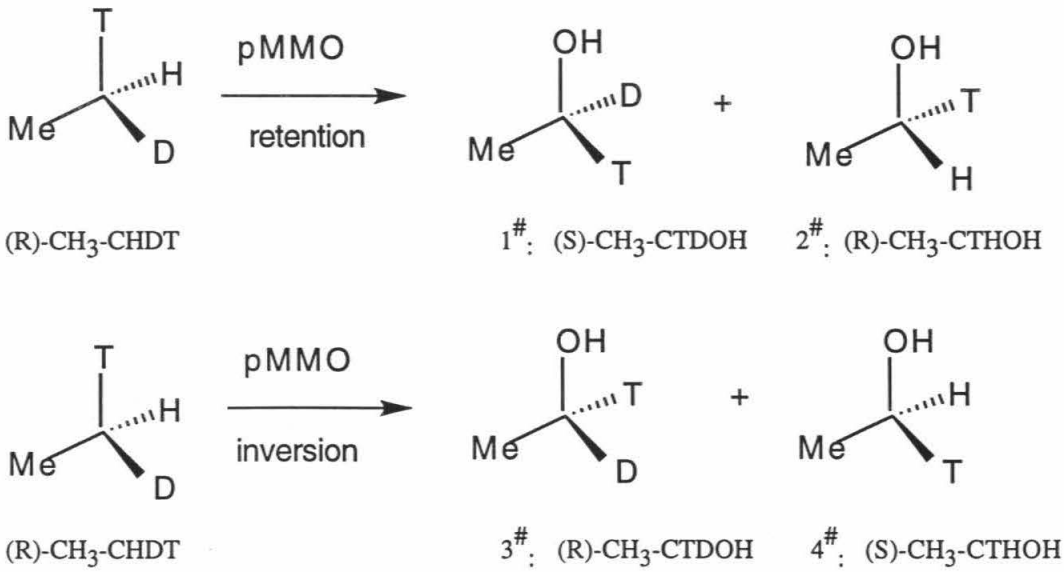
	2-butanol		2-pentanol	
Temperature (°C)	R/(R+S)	ee (%)	R/(R+S)	ee (%)
43	73	46	90	80
38	76	52		
25	79	58		

III. Stereoselectivity of primary alcohols

The carbon atom where the hydroxyl group is attached to in primary alcohol is nonchiral. Isotopically substituted ethane, (S)- or (R)-CH_3-CHDT and (S)- or (R)-CD_3-CHDT, were used as substrates to study the cryptic steric course of alkane hydroxylation catalyzed by pMMO.

Figure 11 is a ^3H NMR spectrum of the reaction mixture from the oxidation of (R)-[1-$^2H_1$,1-$^3H_1$] ethane catalyzed by pMMO. The four peaks shown in the spectrum can be identified as: a) HTO (4. 84 ppm), b) CH_3-CHT-OH (3.67 ppm), c) CH_3-CDT-OH (3. 64 ppm) and d) CHDT-CH_2-OH (1.18 ppm). Another hydroxylation product, CH_3-CHD-OH is silent in the ^3H NMR. The ratio of oxidation at the labeled versus the unlabeled methyl group is about 1/3. The peak integral of b and c determines the intramolecular isotopic effect of this reaction, k_H/k_D~5.0. With tri-deuterated substrate, the ratio of hydroxylation at the labeled versus the unlabeled methyl group changed to ~2.5/1.

The absolute configurations of products b and c were determined and their ratios were used to calculate the stereoselectivity of this reaction. The stereosepecific products produced from hydroxylation of (R)-CH_3-CHDT are shown in the following two chemical equations.



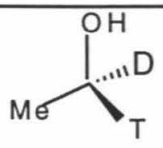
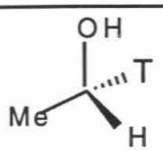
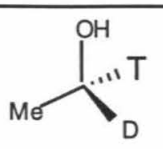
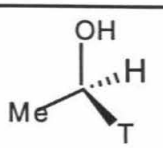
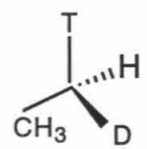
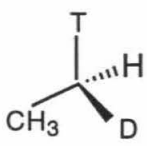
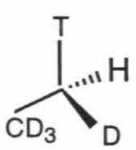
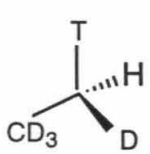
(R)-CH_3-CHDT $1^{\#}$: (S)-CH_3-CTDOH $2^{\#}$: (R)-CH_3-CTHOH

(R)-CH_3-CHDT $3^{\#}$: (R)-CH_3-CTDOH $4^{\#}$: (S)-CH_3-CTHOH

# indicates that the numbering scheme here corresponds with that in Figure 6.

Figure 12 is the ^3H NMR spectrum of the (R)-2-acetoxy-2-phenylethanoate derivative of ethanol recovered from the incubation of (R)-CH_3-CHDT with pMMO. Product distribution is summarized in Table V. Products $1^{\#}$ and $2^{\#}$ were predominately formed (96%) for hydroxylation of (R)-[1-$^2H_1$,1-$^3H_1$] ethane, while products $3^{\#}$ and $4^{\#}$ were the major products (94%) for hydroxylation of (S)-[1-$^2H_1$,1-$^3H_1$] ethane. The same trend was observed for oxidation of CD3-CHDT. Considering that the chiral ethane substrate was only 88% enantiomeric pure, the enantiomeric excess for both of these reactions was 100%. In another words, the hydroxylation of chiral ethane catalyzed by pMMO proceeds with 100% of retention of configuration.

Table V. Percentage of distribution of ^3H label in the methylene group of the (2R)-2-acetoxy-2-phenylethanoate derivatives of the samples generated by pMMO.

product / substrate				
 (R)	1022.1	218.2	45.7	10
 (S)	71.6	10	1000	197.5
 R	198.06	35.46	3.86	10
 (S)	10	21.06	471.5	122.88

IV Tritium water problem

In the studies determining the stereoselectivity of primary alcohol formation using chiral ethane, we have observed the formation of an abnormally large amount of tritium water (HTO). The ratio of HTO/ alcohol varied from run to run (from ~52/48 to 86/14), and it did not have any correlation with the incubation time, nor the amount of inhibitor present in the reaction mixture. The following are the possible processes that could lead to the formation HTO

1. Formation of CH3-CHD-OH

The tritium atom in the hydroxyl group exchanges with the solvent proton rapidly and HTO is thus formed. However, as has been discussed in part III, the formation of CH3-CHD-OH must be less than 1/9 of the total alcohols produced in the system due to the normal isotopic effect we have observed. C-T bond is the least likely to be broken. So HTO generated from this process should be < 1/9 of the total alcohols.

2. Further oxidation of ethanol

The addition of formaldehyde can effectively prevent the further oxidation of the ethanols for a limited period of time. This time period depends on the amount of formaldehyde added into the reaction mixture. The incubation time for the oxidation of chiral ethane oxidation varied from 8 to 30 minutes, which is within the time period that added amount of formaldehyde (6 μM) effectively inhibits the oxidation of ethanol. Assuming that inhibition was not perfect, a certain amount of ethanol was still being further oxidized. The following reactions proceeded:

a). $CH_3\text{-}CHT\text{-}OH \xrightarrow{k_{af}} CH_3\text{-}CHO$

b). $CH_3\text{-}CDT\text{-}OH \xrightarrow{k_{bf}} CH_3\text{-}CHO$

c). $CHDT\text{-}CH_2\text{-}OH \xrightarrow{k_{cf}} CHDH\text{-}CHO$

Reaction a and b would have led to the formation of tritium water. However, we can predict, from the isotopic effect, that $k_{cf} > k_{bf} > k_{af}$. So the product distribution of these three alcohols would have changed with incubation time and inhibitor concentration. We would also expect a smaller intramolecular isotopic effect k_H/k_D for longer incubation time. However, we observed almost identical product distribution [(a+b)/c~1/3] and isotopic effect in three different runs. The fact that we failed to observe a peak corresponding to CHDT-CHO also indicates that the further oxidation process did not occur and was not responsible for the tritium water observed.

The possibility of further oxidation of the product to H^3HO and CO_2 was examined by incubation of the membrane with [1-^{14}C] ethanol.[22] This produced no appreciable $^{14}CO2$.

3. Fast proton exchange between the ethane and the solvent

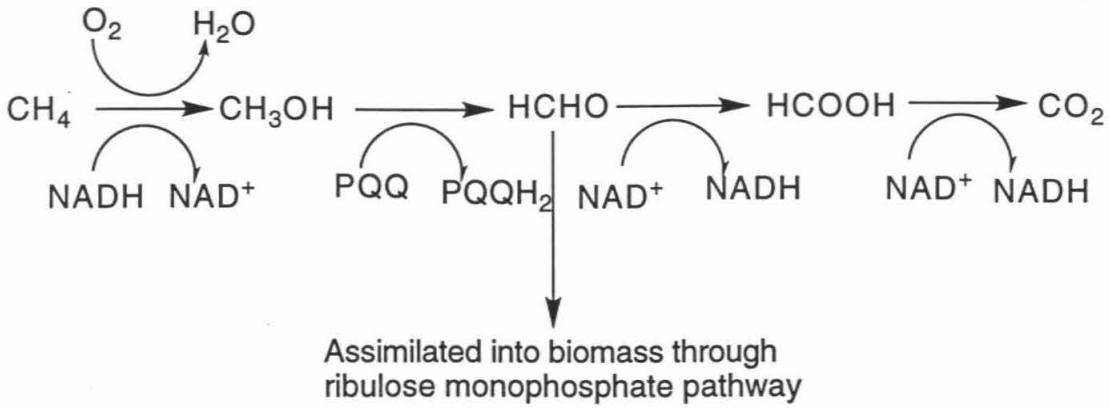
Proton exchange between relatively inert alkane and solvent water is impossible by itself. However, this process might be possible when enzymes are present. This seems to be the only process that leads to the formation of a large amount of tritium water. Unfortunately, this hypothesis is also inconsistent with the experimental results. If proton exchange was facile between alkane substrate and the solvent water, it should be more so between the alcohol and the solvent. CH3-CDT-OH would have been converted to CH3-CHT-OH, and we would have observed a reverse isotopic effect if the exchange process did occur.

A sample of the ethanol produced from an incubation of (S)-[1-$^2H_1$, 2-$^2H_3$] ethane was converted to acetate and purified. Configurational analysis after dirivatization revealed a close enatiomeric excess to the starting substrate.[22] This indicates that proton exchange process does not occur during pMMO mediated alkane oxidation.

The ratio of tritium water/ alcohols varied from run to run, but the stereoselectivity remained the same. So the formation of tritium water does not interfere with the steric pathway of ethane oxidation.

V. Electron transport in the whole cells and membrane extracts

The methane monooxygenase reaction requires two substrates and an electron donor. the following is the methane assimilation and the electron transport pathway in the whole cells[20]:



Many electron donors, such as methanol, formaldehyde, and formate, can be used to supply electrons for the pMMO in the whole cells. Sodium formate was used in our study. Sodium formate stimulates co-oxidation, presumably through its conversion to carbon dioxide, with the concomitant formation of NADH by the formate dehydrogenase. In contrasts, only NADH is accepted as the electron donor when membrane extracts are used as the pMMO source. NADH is converted to NAD^+ in the first oxidation step, but unlike the whole cell system, no processes are know to convert NAD^+ back to NADH is the membrane system. The electrons seem to flow from the alcohols to oxygen when alcohols are oxidized. The details of the electron transfer chain are unknown at present. More research is needed to understand this problem.

V. Mechanistic Implications

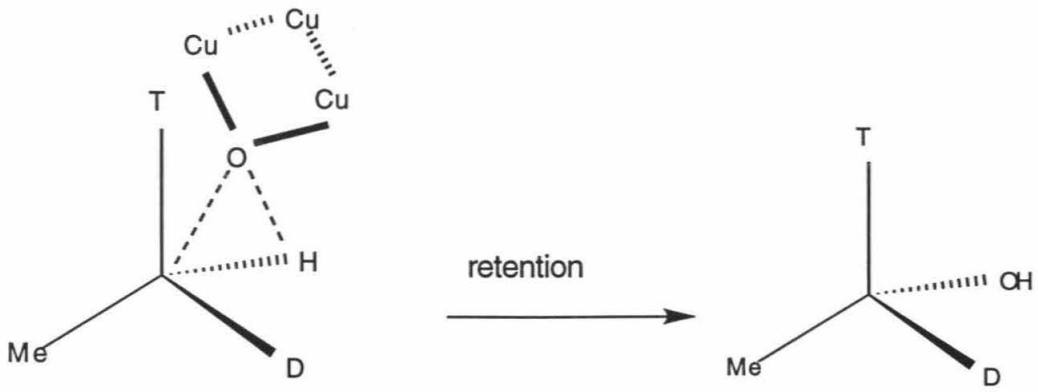
It is obvious from all the data presented in this report that the mechanisms of alkane oxidation catalyzed by pMMO are very complicated. The active sites of pMMO are very small, which explains its very narrow substrate range. This tight binding pocket is probably responsible for the high stereoselectivity observed in the formation of secondary alcohols and isotopically substituted ethanol.

The observation, that significant levels of both (R)- and (S)-2-alcohols are found in the product mixtures from oxidation of n-butane and n-pentane, suggests the concerted mechanism, i.e. the direct insertion of the activated oxygen atom into the substrate

secondary C-H bond, an unlikely scenario. Our experimental data on 2-butanol and 2-pentanol are most consistent with a substrate radical mechanism. A radical intermediate usually gives rise to racemic products due to rapid configurational inversion at the "trigonal" carbon. Since a bias toward the (R)-alcohols was observed, however, other factors must be at work here. The active site of pMMO is presumably small enough to pose a significant kinetic barrier for the substrate radical to undergo configurational inversion, or that one configuration is greatly favored thermodynamically over the other. Increasing the reaction temperature increases the internal energy of the substrates, which in turn could allow a greater population of the radical intermediates to cross the energy barrier, or populate the higher energy configuration in the binding pocket. The observed higher enantioselectivity at lower temperatures is in agreement with such a model. Larger substrates, which fit sluggishly into the active site pocket, will experience greater constraints than the smaller ones; this increases the energy of the thermodynamically less favorable configuration, as well as increases the energy barrier for configurational inversion for the substrate radical intermediate. It also slows down V_{max} of the reaction. The significant increase in the enantioselectivity observed for the hydroxylation of n-pentane can be understood in this light. This dramatic enhancement in enantioselectivity is consistent with the expectation that n-pentane fully occupies the active site of the pMMO.

Ethane is a much smaller substrate than butane and pentane, and it experiences less constrain than the large substrates do. Therefore, a small stereoselectivity was expected. However, our experimental data indicated 100% retention of configuration in ethane oxidation. We propose a slightly different steric pathway in the formation of primary alcohols. The terminal carbons are much less steric hindered than the secondary alcohols, so the activated oxygen can get closer to the terminal C-H bond. We propose a triangular active intermediate in the process of breaking the C-H bond. The breaking of the C-H bond and the formation of a C-O bond occur simultaneously, and the reaction proceed with the retention of configuration. Scheme I illustrates such a process:

Scheme I. The formation of a three center intermediate in the process of primary alcohol formation.



(R)-CH_3-CHDT (S)-CH_3-CDT-OH

Future Research

The membrane associated methane monooxygenase (pMMO) is a very different enzyme from the soluble methane monooxygenase (sMMO), in its active site, its substrate specificity, stereospecificity, and probably electron pathway. Much more research is needed, on the purified protein, to understand the mechanisms of this enzyme. Meanwhile, genetically engineering pMMO to explore its potential industrial application in asymmetric synthesis will also be an interesting direction to go.

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18. The first letter denotes the chirality of the acid and the second letter refers to the chirality of the alcohols in the ester product.

19. The corresponding diastereoisomers of 3-buten-2-ol are not stable on the HPLC column used here. In this case, the enantiomeric excess was determined by the NMR method only.

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Fig. 1. ^{1}H NMR spectrum of (S)-2-acetoxy-2-phenylethanoate derivative of racemic 2-butanol.

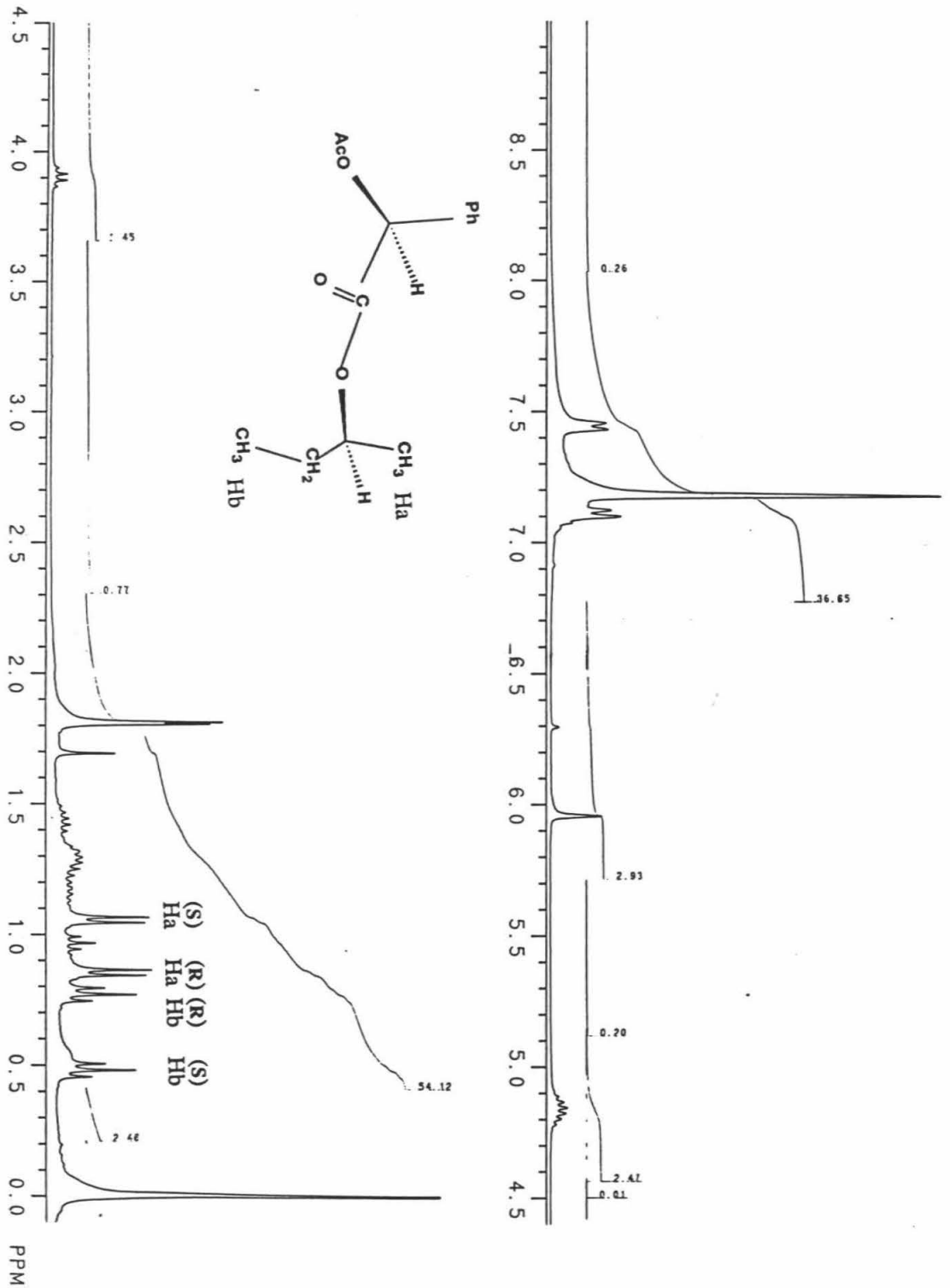
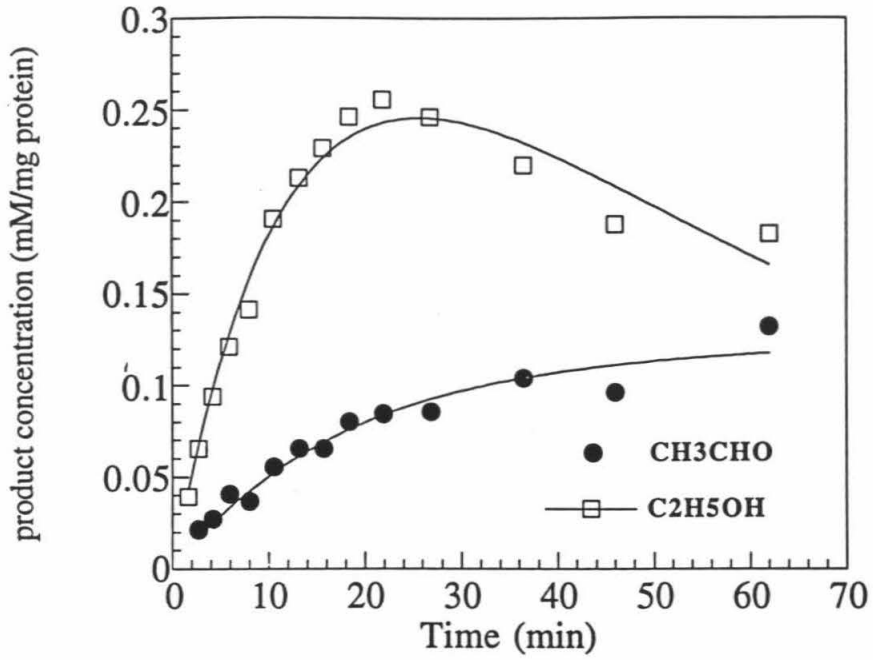
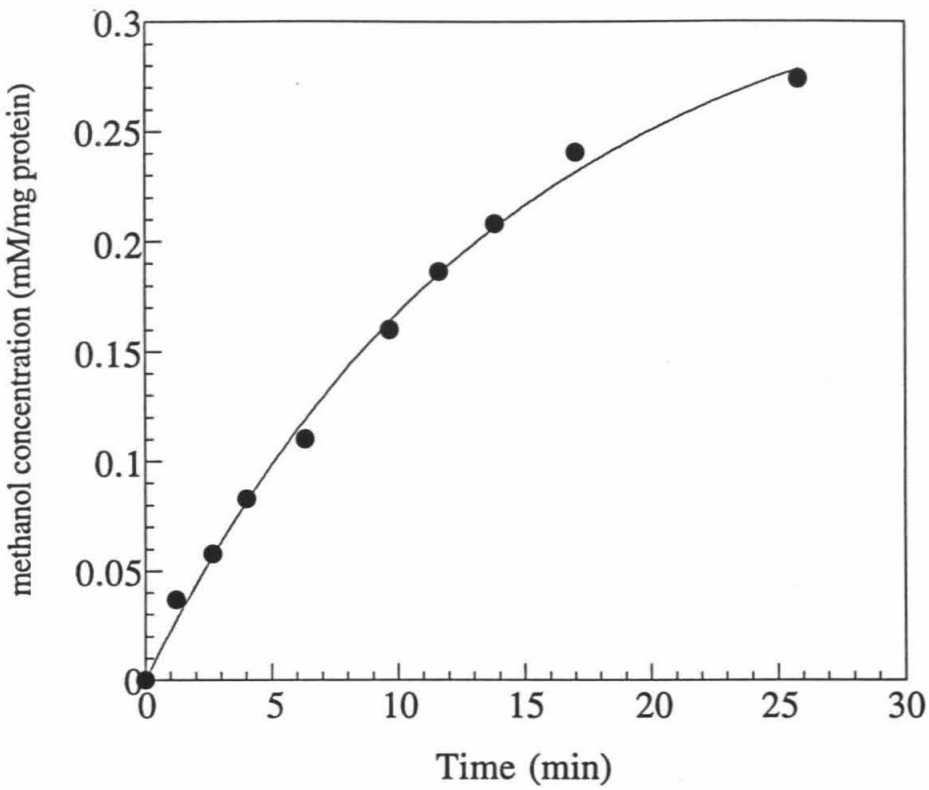


Fig. 2. Ethanol and acetaldehyde formation from oxidation of ethane catalyzed by pMMO.



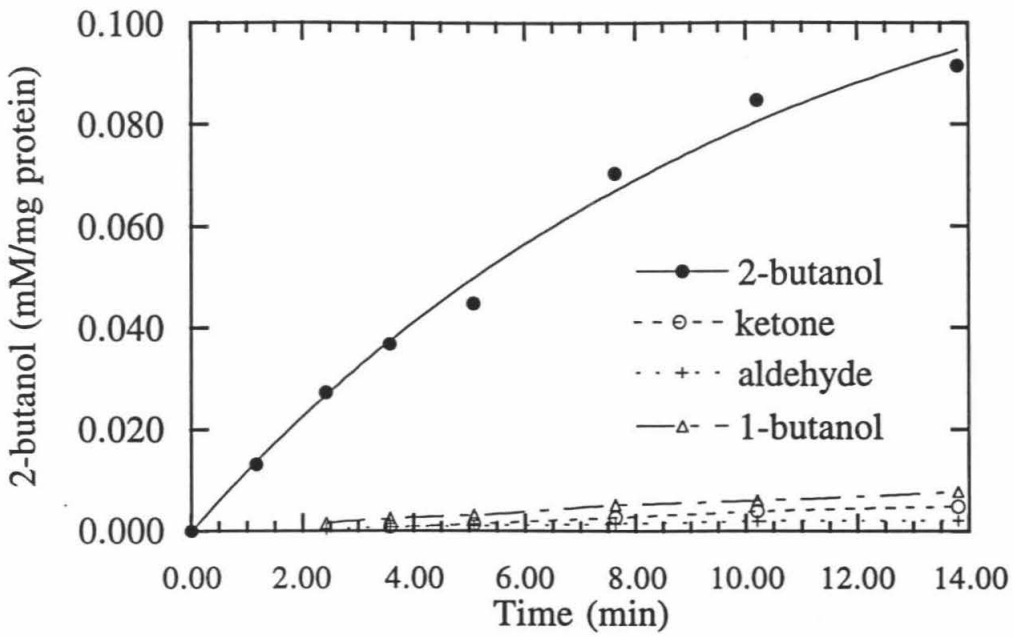
y = M3*m1*(exp(-m1*t)-exp(-m2*t))/(m2-m1)		
	Value	Error
m1	0.073055302019	0.0151913
m2	0.017555251452	0.0051242
m3	0.38568604241	0.0595634
Chisq	0.0015752363038	NA
R	0.98772074234	NA

Fig. 3. Methane oxidation catalyzed by pMMO.



y = m1*(1-exp(-m2*m0))		
	Value	Error
m1	0.33316519549	0.0145136
m2	0.069942173207	0.00535483
Chisq	0.00028470269445	NA
R	0.99813285168	NA

Fig. 4. 2-butanol formation from oxidation of n-butane catalyzed by pMMO.



y = m1*(1-exp(-m2*m0))		
	Value	Error
m1	0.12880335193	0.013148
m2	0.096328652776	0.0158163
Chisq	6.4080462216e-05	NA
R	0.99588934069	NA

Fig. 5. n-pentane oxidation catalyzed by pMMO (0.1 g membrane extracts)

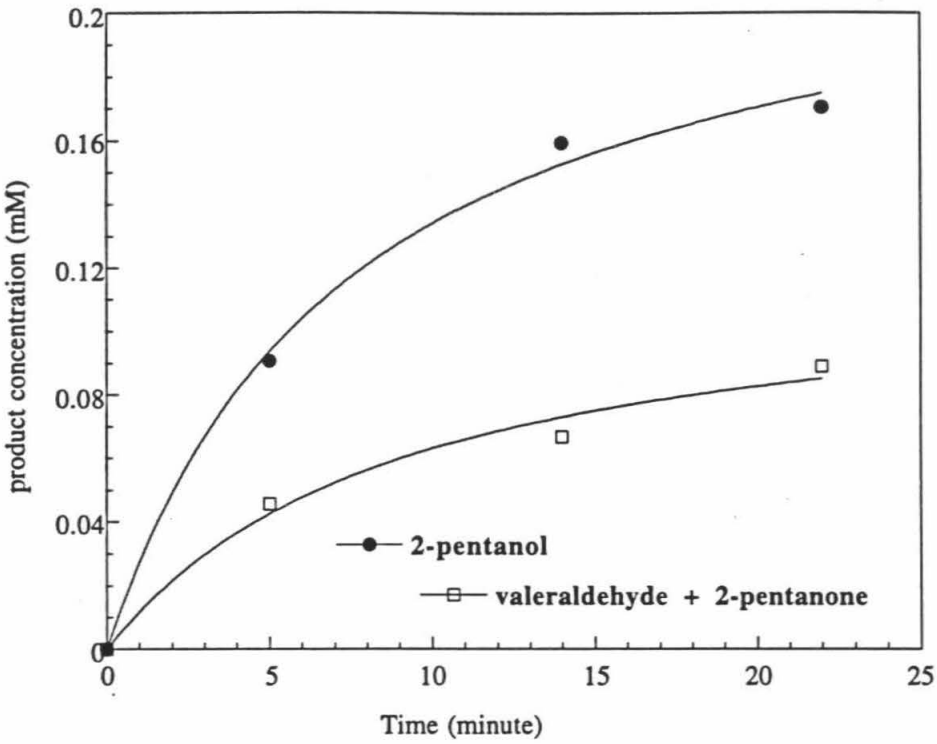


Fig. 6. Ethane oxidation catalyzed by pMMO in the presence of formaldehyde (6 mM)

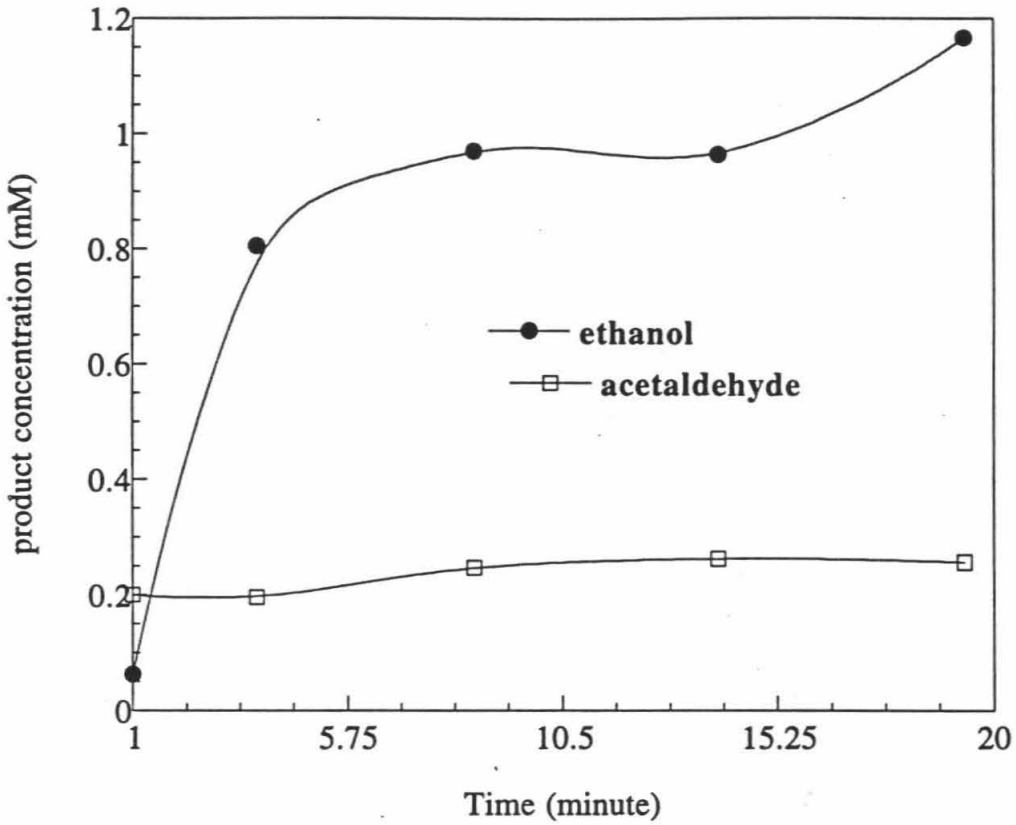


Fig. 7. Ethanol formation from ethane oxidation catalyzed by pMMO in the presence of different amount of formaldehyde.

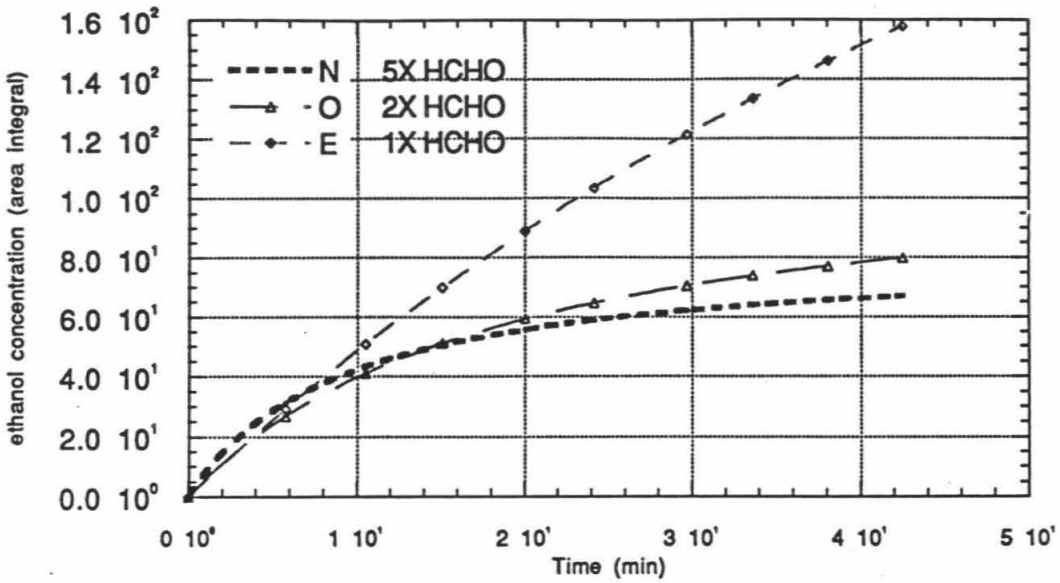


Fig. 8. Oxidation of 1-butanol catalyzed by membrane at different conditions:

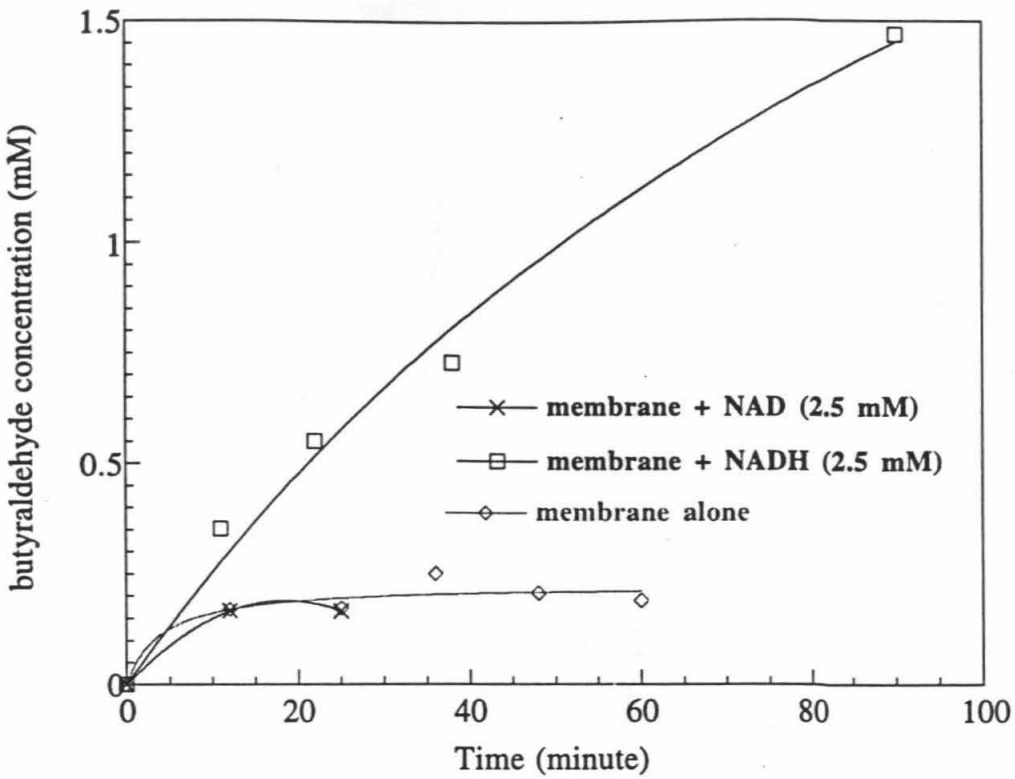


Fig. 9. ^{1}H NMR spectrum of (S)-2-acetoxy-2-phenylethanoate derivative of 2-butanol produced by oxidation of n-butane catalyzed by pMMO.

pMMO source: whole cells; reaction temperature 43 °C

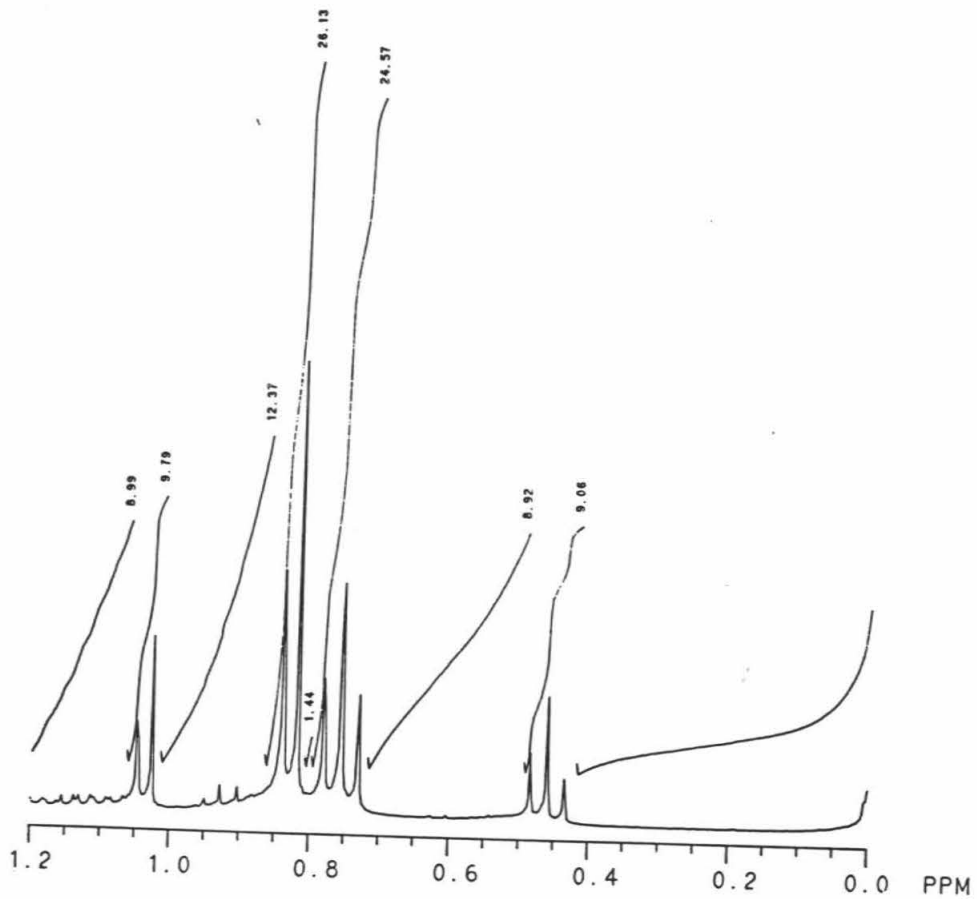


Fig. 10. ^{1}H NMR spectrum of (S)-2-acetoxy-2-phenylethanoate derivative of 2-butanol produced by oxidation of n-butane catalyzed by pMMO.

pMMO source: membrane extracts; reaction temperature 45 °C

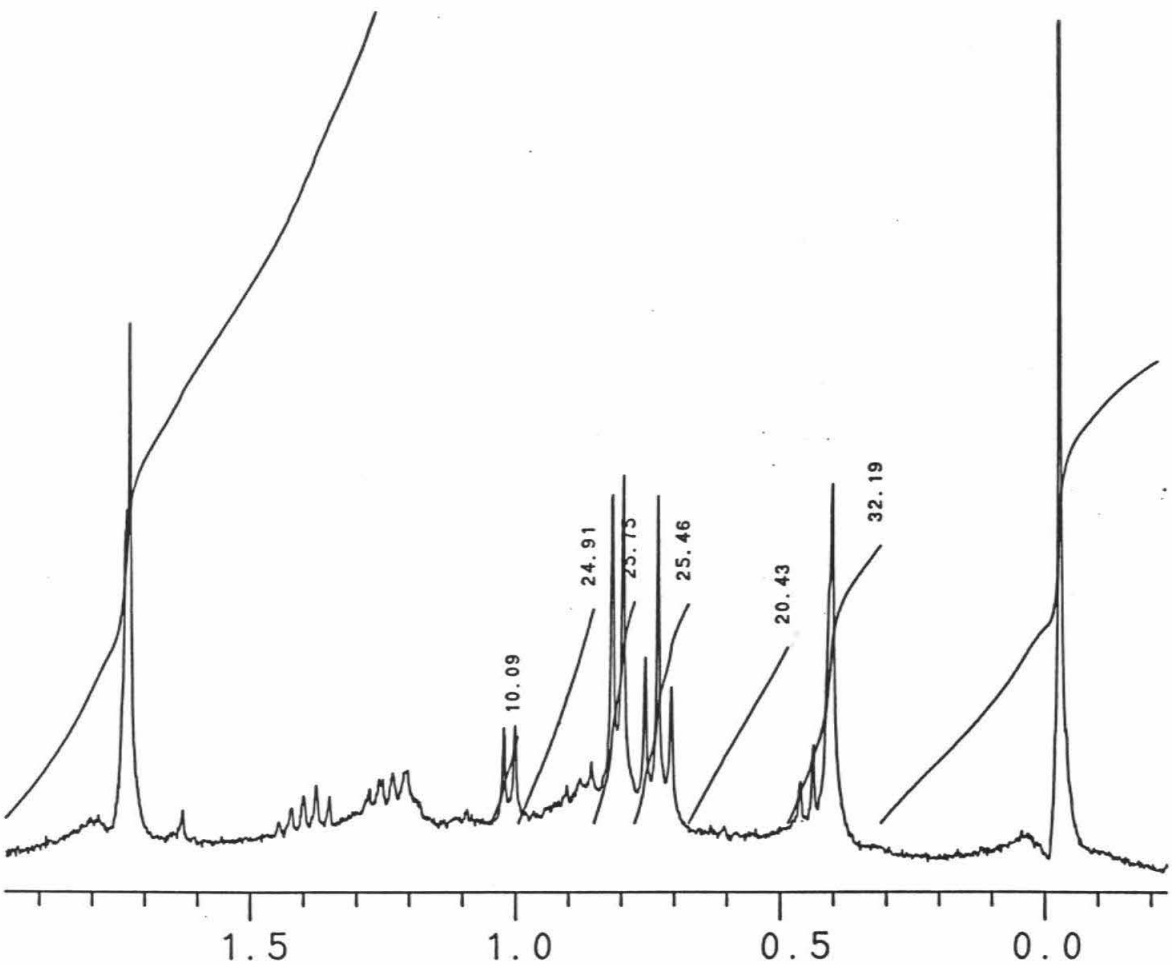


Fig. 11. ^{3}H NMR spectrum of reaction mixture from oxidation of (R)-[1-$^{2}H_1$, $^{3}H_1$] ethane catalyzed by pMMO, at 45 °C.

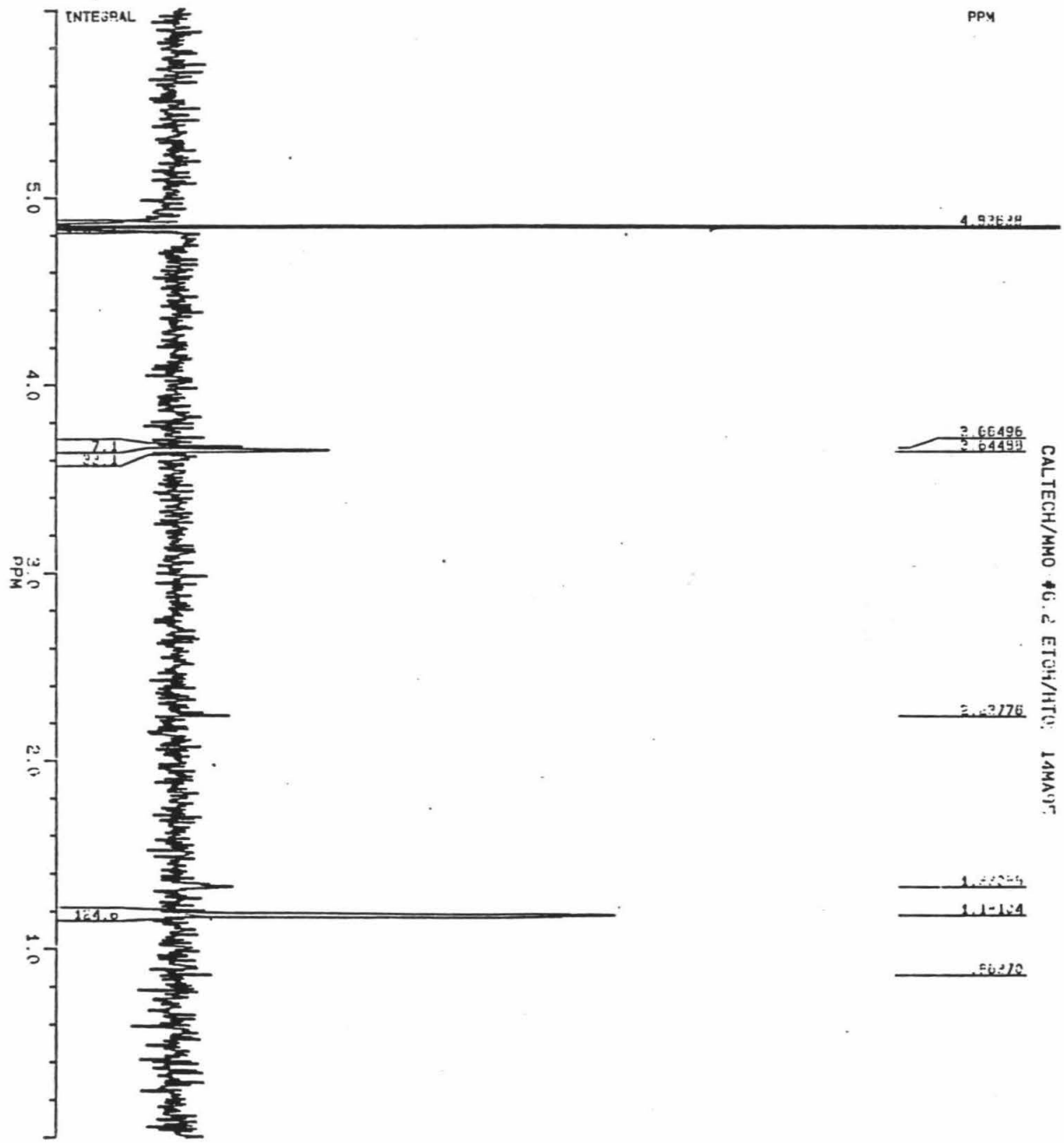


Fig. 12. ^{3}H NMR spectrum (320 MHz, 1H decoupled) of the (2R)-2-acetoxy-2-phenylethanoate derivative of ethanol recovered from the incubation of (R)-[1-$^{2}H_1$, $^{3}H_1$] ethane with pMMO.