# Studies on the Mechanism of Action of Methoxatin-Requiring Methanol Dehydrogenase: Reaction of Enzyme with Electron-Acceptor Dye†

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ABSTRACT: Bacterial methoxatin-dependent methanol dehydrogenase requires an electron acceptor, e.g., phenazine methosulfate (PMS), for activity. Oxidation of methanol shows a deuterium isotope effect of 4.3 in the presence of high concentrations of PMS; the effect is reduced at low PMS concentrations. This suggests that the catalytic reaction comprises at least two steps, one involving substrate and the other PMS. The UV–visible spectrum of methanol dehydrogenase undergoes a series of characteristic changes when PMS is added to the enzyme. It is proposed that these changes correspond to the formation of an enzyme intermediate, resulting from the reaction of PMS with the enzyme, which can subsequently react with substrate. Preincubation of the enzyme with PMS followed by dilution into a solution containing [$^{14}$C]methanol results in an additional enzyme turnover compared to the control performed without preincubation with PMS. This confirms the proposal that the enzyme reacts with PMS before reacting with substrate. The reaction between the enzyme and PMS is not a redox reaction since no oxygen is consumed during the reaction nor is the PMS reduced. Methanol dehydrogenase is inactivated by cyclopropanol in the presence of PMS. The intermediate formed by reaction of the enzyme with PMS is also an intermediate in the cyclopropanol inactivation reaction. Preincubation of the enzyme with PMS gives an increase in the rate of inactivation by cyclopropanol. Cyclopropanol stoichiometrically equivalent to 13% of the enzyme is sufficient to completely inactivate the enzyme. The inactivation reaction shows neither a deuterium nor a tritium isotope effect. When methanol dehydrogenase is inactivated with a mixture of [1-$^{14}$C]cyclopropanol and [1-$^{3}$H]cyclopropanol, 85–95% of the radioactivity can be isolated as a modified form of the cofactor, presumably an adduct between a molecule derived from cyclopropanol and the cofactor. The UV–visible spectrum of this cofactor species suggests that it is a C-5 adduct of methoxatin.

Methanol dehydrogenase, containing the cofactor methoxatin, has been found in several bacteria that grow on $C_1$ compounds. In vitro the enzyme oxidizes primary alcohols to the corresponding aldehyde in the presence of an electron acceptor (e.g., phenazine methosulfate, PMS) and the activator $NH_4^+$. It is maximally active at pH 9.0–9.5 (Yamanaka, 1981).

The enzyme has a stable free-radical signal that arises from the semiquinone form of the cofactor (Westerling et al., 1979; de Beer et al., 1979; Duine et al., 1978). The majority of methanol dehydrogenases have a molecular weight of approximately 120 000 and are composed of two identical subunits (Yamanaka, 1981). One molecule of cofactor in the quinone form and one molecule in the quinol form can be isolated per enzyme molecule. It is proposed that these two forms of the cofactor interact in the enzyme to give the semiquinone (Duine et al., 1981). There are, however, a few methanol dehydrogenases that are monomers of molecular weight 60 000 (Patel et al., 1978). These enzymes have a free-radical signal; however, they must also contain cofactor molecules in different redox states as the level of free radical is not stoichiometric with the amount of enzyme (Mincey et al., 1981). Despite the differences in subunit composition, the two types of dehydrogenase appear to be functionally very similar.

Little is currently known about the mechanism of methanol dehydrogenase or the role of methoxatin in catalysis. Enzyme from which methoxatin has been removed is catalytically inactive (Patel et al., 1978). The enzyme functions only with electron acceptors that will transfer single electrons (Duine et al., 1978); however, the relative importance of the oxidized and semiquinone forms of the cofactor is uncertain. There is evidence that the mechanism involves at least two steps. Mincey et al. (1981) observed a deuterium kinetic isotope effect for the oxidation of methanol, which was reduced at low electron acceptor concentrations. Duine & Frank (1980a) have found ping-pong kinetics for substrate and electron acceptor. Possibly the two steps are reactions of the substrate with an oxidized form of the enzyme to give product, followed by reoxidation of the enzyme by the electron acceptor, or vice versa.

There is no strong evidence for an initial reaction between enzyme and substrate in the absence of electron acceptor. The UV–visible spectrum of enzyme-bound methoxatin does not alter when substrate is added to the enzyme (Anthony & Zatman, 1967). Mincey et al. (1981) found no exchange of substrate C-1 hydrogens with solvent in the absence of electron acceptor nor transfer of these hydrogens to another substrate in the presence of product. It is possible to explain these results in terms of an initial reaction between enzyme and substrate only if the substrate C-1 hydrogen is initially transferred to the enzyme and is not exchangeable with solvent and if the product is not released until after reaction of the enzyme with electron acceptor.

Any reaction between enzyme and electron acceptor is difficult to investigate as the enzyme is inactivated by the electron acceptor in the absence of substrate. Duine and co-workers, investigating one of the methanol dehydrogenases that is a dimer, have suggested that the first reaction is in fact one between enzyme and electron acceptor. They propose a cycle (Scheme II) where the enzyme ($MDH_{ox_1}$, semiquinone form) undergoes one-electron oxidation to the quinone form ($MDH_{ox}$*); this then reacts with the substrate to give product and the cofactor in the quinol form ($MDH_{red}$), and finally the

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cofactor is reoxidized ($MDH_{ox_1}$) (de Beer et al., 1983). Presumably the subunits act together since, in contrast to $MDH_{ox_1}$, $MDH_{ox}$* and $MDH_{red}$ have no free-radical signal (Duine & Frank, 1981).

Methanol dehydrogenase is inactivated by cyclopropanol in the absence of electron acceptor (Mincey et al., 1981). The inactivation showed a deuterium kinetic isotope effect of 4. It was suggested that cyclopropanol is oxidized by the enzyme and that reaction of the enzyme with the oxidized product leads to inactivation; however, the exact nature of the products of the inactivation was not determined.

This paper describes an investigation of the methanol dehydrogenase from *Methylomonas methanica* (Patel et al., 1978). This enzyme is one of the monomeric dehydrogenases. The reactions between enzyme and substrate, and between enzyme and electron acceptor, have been examined in order to elucidate the reaction sequence. The interaction of cyclopropanol with the enzyme has also been studied.

## Experimental Procedures

*Chemicals.* Sodium borohydride, 1,3-dichloroacetone, dimethyl sulfoxide, and [$^2H_4$]methanol were from Aldrich, [$^2$H]$NaBH_4$ was from Merck, PMS was from Sigma, 5,5-dimethyl-1,3-cyclohexanedione was from Eastman, and other chemicals were from Fisher. [2-$^{14}$C]Glycerol and [$^3$H]$NaBH_4$ were from New England Nuclear.

*Enzyme Purification.* The procedures for growing *M. methanica* and for purifying the methanol dehydrogenase from the cell paste were as described by Mincey et al. (1981) and Patel et al. (1978) with the following modifications. The protamine sulfate solution was prepared in 0.1 M sodium borate, pH 9.0, rather than in tris(hydroxymethyl)aminomethane (Tris) base. A Sephadex G-150 column was used in place of the Bio-Gel agarose A-1.5 column for the gel filtration step. Instead of recrystallization from an ammonium sulfate solution, the final purification was achieved by loading the protein onto a hydroxyapatite column (11 × 2.7 cm) equilibrated in 50 mM sodium phosphate, pH 7.0, washing the column with 100 mL of 200 mM sodium phosphate, pH 7.0, and eluting the enzyme with 500 mM sodium phosphate, pH 7.0. The enzyme so obtained had a specific activity of 2.5 IU/mg.

The enzyme (50–100 mg/mL) was stored in 50% (v/v) glycerol and 50% (v/v) 50 mM sodium phosphate, pH 7.0, at −80 °C. Before use, the enzyme preparation was thawed at 5 °C and dialyzed extensively against 50 mM sodium phosphate, pH 7.0. Enzyme concentrations were determined by using $E^{0.1\%}_{280nm}$ = 0.98 (Mincey et al., 1981) and also by colorimetric techniques (Lowry et al., 1951).

*Enzyme Assay.* The enzyme was assayed in an oxygen electrode by observing the reduction of oxygen. The standard assay was performed at 25 °C in a thermostated cell in 0.4 mL of 0.3 M sodium borate, pH 9.0, 40 mM $NH_4Cl$, 8 mM methanol, and 4 mM PMS. The assay was initiated by the addition of enzyme. The initial rate of oxygen reduction was obtained from the recorder trace. One unit of enzyme is defined as the amount required to reduce 1 μmol of oxygen to hydrogen peroxide per minute at 25 °C.

*Absorption Spectra.* The spectra were obtained on a Perkin-Elmer 559 UV–visible spectrophotometer with 1-cm quartz cells at 25 °C. The same instrument was used to record absorbance changes with time at a single wavelength.

*Electron Spin Resonance Spectra.* Electron spin resonance spectra (ESR) were obtained on a Varian E-4 electron spin resonance spectrometer at −160 °C. Standard experimental parameters were as follows: time constant 1 s; modulation amplitude 2 G; receiver gain 8 × $10^3$; microwave power 2 mW; field set 3240 G; microwave frequency 9.149 GHz. The ESR spectra were kindly recorded by A. S. Mildvan.

*Determination of Radioactivity.* Samples were dissolved in 4 or 10 mL of aqueous counting scintillant (Amersham) and counted in a Beckman LS 1800 liquid scintillation system. The counting efficiency in the case of samples containing both carbon-14 and tritium was determined as follows. Duplicates of each sample were counted. Then a tritium standard was added to one and a carbon-14 standard to the other, and the samples were recounted. The standards were radiolabeled toluene samples from New England Nuclear.

*High-Pressure Liquid Chromatography (HPLC).* Two HPLC systems were used in this study. One was an isocratic system using a Bio-Rad Aminex ion-exclusion HPX 87H column (300 × 7.8 mm) in 5 mM sulfuric acid. The column was pumped at 0.6 mL/min with a Laboratory Data Control minipump. The sample was introduced into the system via a Rheodyne injector with a 1-mL loading loop. The eluent was analyzed by using a Waters Associates R401 differential refractometer. This system was used to separate methanol, formaldehyde, and formate, the components in the product distillate from the pulse–chase experiments. The following retention times were observed: formaldehyde, 7.5 min; formate, 8.5 min; methanol, 11.5 min. The system was also used in trying to identify the small molecular weight component released from the cofactor adduct isolated from cyclopropanol-inactivated enzyme.

The second HPLC system was composed of the following components from Beckman Instruments: two 112 solvent delivery modules connected to a 340 organizer with a series 210 universal sample injection valve (2-mL loading loop) and coordinated by a 420 controller. The column was a Waters Associates μBondapak $C_{18}$ column (300 × 7.8 mm). The column eluent was monitored at 254 nm by using a Beckman 8-μL analytical UV detector, Model 153. This system was used to separate the cofactor species isolated from cyclopropanol-inactivated enzyme. The sample was loaded onto the column equilibrated at 1 mL/min in 0.5% (w/v) ammonium acetate and the column washed under these conditions. After 30 min a 30-min linear gradient of 0–40% (v/v) methanol (HPLC grade) in 0.5% (w/v) ammonium acetate was started. Finally, the column was washed for 10 min with 0.5% (w/v) ammonium acetate containing 40% (v/v) methanol. The absorbance at 315 nm of fractions collected during the elution was measured subsequently to ensure that no products were overlooked.

The Beckman system was also used isocratically with an Altex 156 refractive index detector to purify samples of cyclopropanol. The $C_{18}$ column was equilibrated with water at 2 mL/min. Cyclopropanol had a retention time of 3 min.

*Reaction of Formaldehyde with 5,5-Dimethyl-1,3-cyclohexanedione.* Formaldehyde was derivatized essentially as described by Schneider (1946). A neutral aqueous solution of 5,5-dimethyl-1,3-cyclohexanedione was prepared (28 mM) and added at 10-fold molar excess to the samples containing radioactive formaldehyde. The solution was stirred overnight at room temperature. The white crystals that formed were recrystallized from ethanol to constant specific activity by using careful evaporation.

*Syntheses. (A) [1-$^{14}$C]Cyclopropanol.* [1-$^{14}$C]Cyclopropanol was prepared from [2-$^{14}$C]glycerol by first converting the glycerol to 1,3-dichloro-2-propanol (Conant & Quayle, 1944) and then reacting the 1,3-dichloro-2-propanol with ethylmagnesium bromide in the presence of $FeCl_2$ to give

cyclopropanol (DePuy et al., 1964; J. Sinsheimer, unpublished procedure).

To 100 mg (1.09 mmol) of [2-$^{14}$C]glycerol (250 $\mu$Ci, 0.23 mCi/mmol) in 2.5 mL of water was added 105 mg (1.75 mmol) of glacial acetic acid. HCl gas was bubbled through the solution, maintained at 90–100 °C, for 60 h. Further 105-mg aliquots of glacial acetic acid were added at 24 and 48 h. The solution was cooled on ice, diethyl ether added, and the solution neutralized by the slow addition of solid $NaHCO_3$. Sufficient solid NaCl to give a saturated solution was then added and the solution stirred at 4 °C for 5 h. The ether layer was removed and the aqueous phase extracted with a second aliquot of ether. The ether layers were combined and left over anhydrous sodium sulfate overnight.

Ethylmagnesium bromide (1.5 mL, prepared by the reaction of 0.4 g of magnesium with 1 mL of bromoethane in 5 mL of diethyl ether) was slowly added with stirring to the anhydrous 1,3-dichloro[2-$^{14}$C]propanol containing 7 mg (55 $\mu$mol) of $FeCl_2$ in 4 mL of diethyl ether. The mixture was kept under $N_2$ at room temperature. When the addition was complete, the solution was stirred for 12 h. The ether solution was then added dropwise to a saturated aqueous solution of boric acid at 4 °C. The ether layer was removed and the aqueous layer was extracted twice with ether. Water (2.0 mL) was added to the combined ether layers and the ether evaporated with $N_2$. The aqueous solution containing [1-$^{14}$C]-cyclopropanol was bulb-to-bulb distilled at reduced pressure. The product was further purified and characterized as described below.

(*B*) [*1-$^2$H*]*Cyclopropanol.* [1-$^2$H]Cyclopropanol was prepared by reducing 1,3-dichloroacetone with [$^2$H]$NaBH_4$ (Brown & Ichikawa, 1961) and converting the 1,3-dichloro-[2-$^2$H]propanol thus formed to cyclopropanol. [$^2$H]$NaBH_4$ (23 mg, 0.55 mmol) was slowly added to 2 g (15.8 mmol) of 1,3-dichloroacetone in 20 mL of distilled water at room temperature. The mixture was stirred for 1 h. Additional [$^2$H]$NaBH_4$ (600 mg, 14.3 mmol) was then added and the stirring continued for 3 h. The solution was neutralized by adding 2 N HCl and saturated with solid NaCl. The solution was then extracted 3 times with ether, and the ether layers were combined and dried overnight with solid sodium sulfate. The ether solution of 1,3-dichloro[2-$^2$H]propanol was converted to [1-$^2$H]cyclopropanol as described above for the synthesis of [1-$^{14}$C]cyclopropanol.

(*C*) [*1-$^3$H*]*Cyclopropanol.* This compound was prepared as described for [1-$^2$H]cyclopropanol but with [$^3$H]$NaBH_4$. The 1,3-dichloroacetone (200 mg, 1.6 mmol) was initially reacted with 2.3 mg (0.05 mmol) of [$^3$H]$NaBH_4$ (400 Ci/mol) in 2 mL of water. For the second addition 60 mg (1.43 mmol) of unlabeled $NaBH_4$ was used.

(*D*) *Cyclopropanol.* Unlabeled cyclopropanol was prepared both from nonradioactive glycerol by the procedure used to obtain [1-$^{14}$C]cyclopropanol and from 1,3-dichloroacetone by using unlabeled $NaBH_4$. In experiments where labeled cyclopropanol and unlabeled cyclopropanol were compared, samples synthesized by the same method were used.

The synthetic procedures described above give an aqueous solution of crude cyclopropanol. In each case the cyclopropanol was purified by reverse-phase HPLC, concentrated by saturating the solution with solid NaCl, by extracting with ether, and by reextracting the cyclopropanol with water, and then repurified on HPLC. The cyclopropanol solutions gave a single peak on HPLC, and in the case of the radioactive samples 100% of the radioactivity was associated with the peak. To obtain a proton nuclear magnetic resonance spectrum, the purified cyclopropanol was reextracted from the ether into $^2H_2O$. The spectrum displayed proton resonances at $\delta$ 3.4 (multiplet) and $\delta$ 0.45 (doublet) in agreement with literature values (Martinez et al., 1975).

The concentration of the cyclopropanol in $^2H_2O$ was determined by adding a known amount of dimethyl sulfoxide to the $^2H_2O$ solution and comparing the integral of the proton resonances of the cyclopropanol with that of the dimethyl sulfoxide peak at $\delta$ 2.5 (singlet). The concentrations of the labeled cyclopropanol solutions were found by comparing the peak areas from HPLC traces with the peak area of a solution standardized by $^1$H NMR. For the [1-$^3$H]- and [1-$^{14}$C]-cyclopropanols the concentrations were verified as follows. Methanol oxidase from *Hansenula polymorpha* contains two oxidized flavins and is completely inactivated by cyclopropanol. The stoichiometry of inactivation is 1 mol of cyclopropanol per mole of oxidized flavin (B. Sherry, unpublished observations; Mincey et al., 1980). Thus the concentrations of the radiolabeled cyclopropanol solutions were confirmed by titration of methanol oxidase.

*Isolation and Characterization of Products from Pulse–Chase Experiments.* After the reaction was stopped by addition of $HClO_4$, carrier formaldehyde and formic acid were added to each experiment. The mixtures were then bulb-to-bulb distilled at reduced pressure and the distillates analyzed by HPLC using the Aminex ion-exclusion column. The amount of [$^{14}$C]methanol converted to [$^{14}$C]formaldehyde in each experiment was determined. The recovery of the product in each case was found from the peak area of the HPLC trace. The experiment without enzyme showed that the [$^{14}$C]-methanol contained radioactive material that ran in the region of formaldehyde on HPLC. This contaminant corresponded to only 8% of the total radioactivity. In addition to analyzing the results of HPLC, an aliquot of the product distillate was reacted with 5,5-dimethyl-1,3-cyclohexanedione. The specific activity of the derivative was determined and hence the amount of [$^{14}$C]formaldehyde formed in each experiment calculated. To confirm that the only product formed under these conditions was formaldehyde, an aliquot of the distillate was redistilled at pH 7.5. Only methanol and formaldehyde peaks were observed on HPLC, and the expected amount of radioactivity was associated with the formaldehyde. As a second test, an aliquot of the distillate was reduced with sodium borohydride, redistilled, and rerun on HPLC. In this case methanol and formate peaks were observed, and only the methanol peak was radioactive.

## Results

*Electron Spin Resonance Spectra.* The ESR spectrum of the enzyme in 50 mM sodium phosphate, pH 7.0, was recorded. The signal was quantitated by comparison of the double integral of the signal with the double integral of the signal of a $Cu^{2+}$–ethylenediaminetetraacetic acid standard solution. The radical concentration was found to be 1% of the total enzyme concentration. This is substantially lower than the value of 13% observed by Mincey et al. (1981).

*Isotope Effects for Methanol Oxidation.* The enzyme was assayed in the presence of methanol and [$^2$H]methanol at various PMS concentrations (Table I). The enzyme shows a deuterium kinetic isotope effect for the oxidation of methanol that is reduced at low PMS concentrations. This confirms the results of Mincey et al. (1981).

*UV–Visible Spectrum of Methanol Dehydrogenase.* After storage the enzyme is dialyzed against 50 mM sodium phosphate, pH 7.0. Before the absorption spectrum is recorded, the enzyme is diluted into 0.3 M sodium borate, pH 9.0/40

Table I: Deuterium Kinetic Isotope Effect for Methanol Oxidation[a]

| PMS concn (mM) | $V_{max}^{CH_3OH}/V_{max}^{CD_3OH}$ |
|---|---|
| 4.1 | 4.3 |
| 1.2 | 2.6 |
| 0.4 | 1.5 |

[a] Reaction rates were measured in the oxygen electrode under standard assay conditions, except that the concentration of PMS was varied.

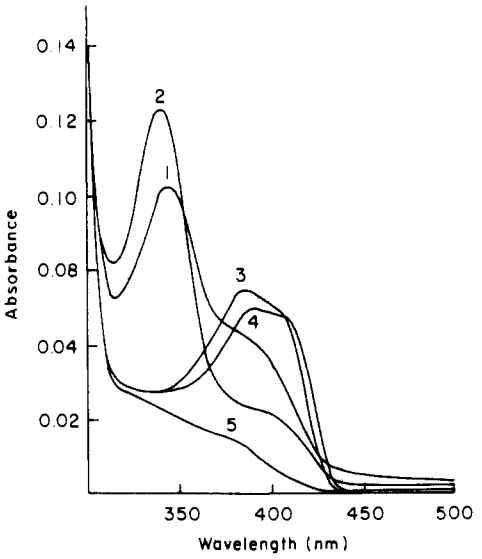



FIGURE 1: Absorption spectra of methanol dehydrogenase. Enzyme (1) alone, (2) with PMS and methanol, (3) with PMS and cyclopropanol, (4) with PMS and potassium cyanide, and (5) with PMS only. Spectra were recorded in 0.3 M sodium borate, pH 9.0/40 mM $NH_4Cl$ with enzyme (22 $\mu$M), PMS (3 $\mu$M), methanol (16 mM), cyclopropanol (1.3 mM), and potassium cyanide (5 mM), at the following times after addition of enzyme: spectra 2 and 4, 4 min; spectrum 3, 12 min; spectrum 5, 35 min.

mM $NH_4Cl$. The absorption spectrum of methanol dehydrogenase immediately after dilution is shown in Figure 1 (spectrum 1). The spectrum gradually changes to that of spectrum 2 with $t_{1/2}$ = 10–25 min. The rate of this spectral change is accelerated by $NH_4^+$. As previously reported (Anthony & Zatman, 1967; Mincey et al., 1981), addition of methanol does not alter the spectrum of the enzyme. However, in the presence of PMS and methanol spectrum 2 is obtained.

Addition of cyclopropanol and PMS to the enzyme gives spectrum 3. A similar spectral change occurs when potassium cyanide and PMS are added to the enzyme (spectrum 4). These changes do not occur in the absence of PMS. The change observed for potassium cyanide can be reversed by methanol (16 mM), giving spectrum 2. The spectrum obtained with cyclopropanol is not affected by adding methanol. If PMS is added to the enzyme alone, spectrum 5 is obtained. In contrast to the results presented here, Mincey et al. (1981) observed no change in the enzyme spectrum on addition of PMS.

*Spectral Changes at 400 nm.* In order to study the time dependence of the spectral changes observed for methanol dehydrogenase, the changes were monitored at 400 nm. It was decided to use this wavelength since there is a relatively large difference in the absorbance of the various forms of the enzyme and also because PMS has a minimal absorbance at this wavelength. The absorbance change at 400 nm observed when PMS is added to the enzyme is shown in Figure 2. There is an initial rapid drop in the absorbance to A, then a slower rise to B, and finally a slow decrease in absorbance to give spectrum 5 in Figure 1. The absorbance at A is that observed in the

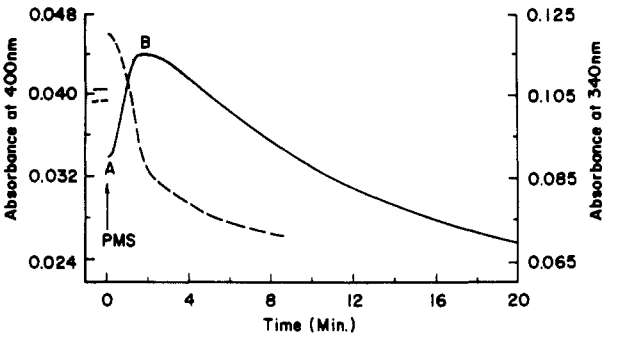



FIGURE 2: Reaction of PMS with methanol dehydrogenase. PMS (1.6 $\mu$M) was added at the point indicated to the enzyme (22 $\mu$M) in 0.3 M sodium borate, pH 9.0/40 mM $NH_4Cl$. Absorbance changes were measured at 400 (—) and 340 nm (– –).

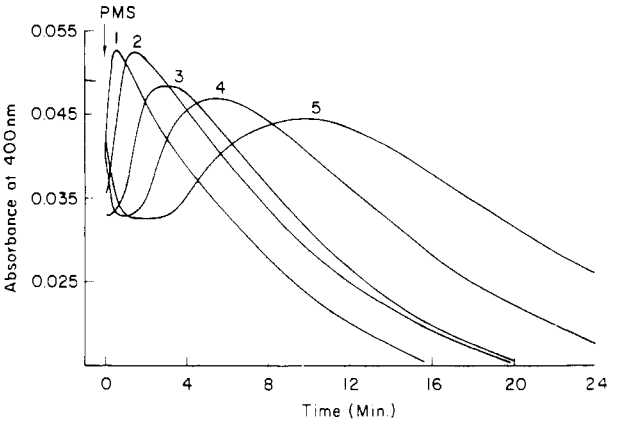



FIGURE 3: Dependence of absorbance changes on various PMS concentrations. PMS was added at the point indicated to the enzyme (25 $\mu$M) in 0.3 M sodium borate, pH 9.0/40 mM $NH_4Cl$. The concentrations of PMS were as follows: (1) 8.15, (2) 1.63, (3) 0.81, (4) 0.33, and (5) 0.16 $\mu$M. The concentrations were determined with an extinction coefficient of 26 300 at 387 nm (Zaugg, 1964). Absorbance was measured at 400 nm.

presence of substrate. For comparison the absorbance change at 340 nm under these conditions is also shown in Figure 2.

The effect of varying the concentration of PMS on the spectral change at 400 nm is shown in Figure 3. At low PMS concentrations there was a lag in the A to B transition proportional to the PMS concentration and a reduction in the height of B. The rate of the A to B transition is dependent on the PMS concentration; however, the decay from B appears to be proportional to the amount of B formed. The effect of varying the enzyme concentration at 8.15 $\mu$M PMS was also investigated. At 75 and 225 $\mu$M enzyme the rate of the A to B transition became too fast to measure; however, the rate constant for the decay from B was unaffected. These results suggest that the A to B transition involves a reaction between enzyme and PMS and that the rate of decay from B depends only on the concentration of B.

The spectral change at 400 nm in the presence of PMS and cyclopropanol is shown in Figure 4. Initially the spectral change was identical with that observed in the presence of PMS alone. However, at the higher cyclopropanol concentrations the absorbance continued to rise at B and then leveled off as expected from Figure 1. For lower concentrations of cyclopropanol the decrease in absorbance after B was observed. The minimum concentration of cyclopropanol required to prevent the decrease in absorbance after B is between 2.4 and 3.7 $\mu$M, which corresponds to 12–17% of the total enzyme concentration. This observation is consistent with the finding that cyclopropanol at 14% of the total enzyme concentration is sufficient to completely inactivate the enzyme (Mincey et

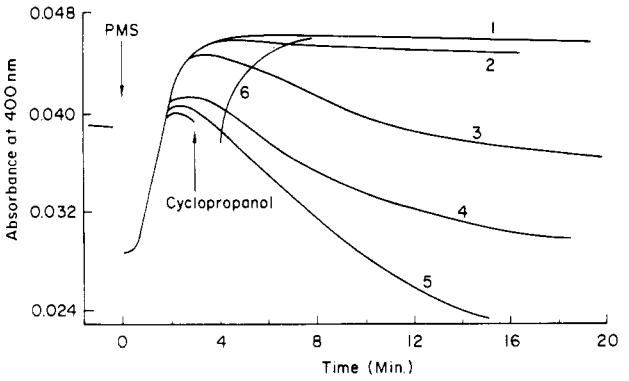



FIGURE 4: Reaction of methanol dehydrogenase, cyclopropanol, and PMS. Various concentrations of cyclopropanol were added to the enzyme (21 $\mu$M) in 0.3 M sodium borate, pH 9.0/40 mM $NH_4Cl$. PMS (3 $\mu$M) was added at the point indicated. The cyclopropanol concentrations were (1) 4.9, (2) 3.7, (3) 2.4, (4) 1.2, and (5) 0.2 $\mu$M. (6) Cyclopropanol was added (4.9 $\mu$M) 3 min after addition of the PMS. Absorbance was measured at 400 nm.

Table II: Effect of Preincubation with PMS on the Inactivation of Methanol Dehydrogenase by Cyclopropanol[a]

| | activity (%) |
|---|---|
| cyclopropanol addition at time | |
| 0 min | 67 |
| 1 min | 39 |
| 2 min | 17 |
| no cyclopropanol | 80[b] |
| no cyclopropanol, no PMS | 100[b] |

[a] Methanol dehydrogenase (20 $\mu$M) in 1 mL of 0.3 M sodium borate, pH 9.0/40 mM $NH_4Cl$ was incubated with PMS (3 $\mu$M) at 25 °C. Cyclopropanol (120 $\mu$M) was added at the times indicated. After a further 1.5 min 10 $\mu$L of the incubation mixture was assayed in the $O_2$ electrode to determine remaining activity. [b] Remaining activity was determined 3.5 min after addition of enzyme to PMS.

al., 1981). In an additional experiment cyclopropanol (4.9 $\mu$M) was added to the enzyme 3 min after the addition of the PMS. As expected, the absorbance rose to the plateau value.

In order to correlate the spectral changes with enzyme activity, the enzyme was incubated with PMS or with PMS and cyclopropanol under conditions identical with those used to follow the spectral changes. At various times aliquots were removed and assayed in the $O_2$ electrode. The results are shown in Figure 5. Inactivation of the enzyme by cyclopropanol in the presence of PMS shows a lag period of approximately 2 min. This suggests that no inactivation occurs until after B in the spectral change. Similarly, the inactivation by PMS alone shows an increasing rate with time, suggesting that the decrease in absorbance after B corresponds to this reaction.

It is possible that B represents a form of the enzyme that has reacted with PMS and that this form can undergo subsequent reaction with cyclopropanol. To test this hypothesis, enzyme was allowed to react with PMS for various times (0–2 min) before adding cyclopropanol. After a further 1.5 min the enzyme was assayed. The results are given in Table II. Preincubation of the enzyme with PMS, which allows the enzyme to be converted to B, leads to greater inactivation by cyclopropanol. This is consistent with the idea that B is an intermediate in the cyclopropanol inactivation reaction.

Form B could also be an intermediate in the normal catalytic cycle of the enzyme. If this were the case, the rate of formation of B must be equal to or greater than the overall rate of methanol oxidation. The rate of the spectral change from A to B for 20 $\mu$M enzyme in the presence of 1.6 $\mu$M PMS is 19 nmol of enzyme converted/min if the total spectral change corresponds to all of the enzyme and 2.7 nmol/min if the total

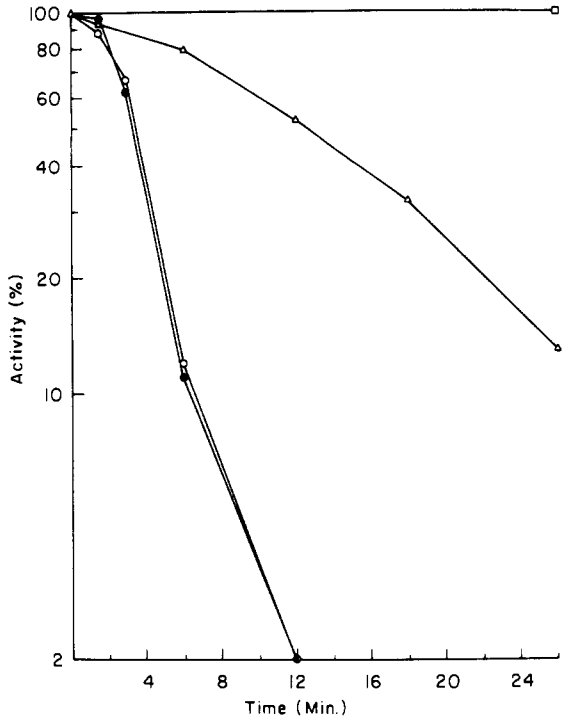



FIGURE 5: Rate of inactivation of methanol dehydrogenase by cyclopropanol and PMS. Enzyme (22 $\mu$M) at 25 °C in 1 mL of 0.3 M sodium borate, pH 9.0/40 mM $NH_4Cl$ alone (□) and enzyme with 2 $\mu$M PMS (△), 2 $\mu$M PMS and 5 $\mu$M cyclopropanol (○), and 2 $\mu$M PMS and 5 $\mu$M [1-$^2$H]cyclopropanol (●). At various times 20-$\mu$L aliquots were assayed in the $O_2$ electrode.

spectral change corresponds to 14% of the enzyme. The rate of methanol oxidation for 20 $\mu$M enzyme was measured in the $O_2$ electrode for a range of PMS concentrations (4.1–30.6 $\mu$M). The data were extrapolated to give a rate of 2.5 nmol of $O_2$/min for 1.6 $\mu$M PMS. Thus B is kinetically competent to be an intermediate in the methanol oxidation reaction.

*Pulse–Chase Experiments.* It appears from the foregoing experiments that the enzyme must react with PMS before it can react with cyclopropanol. The question then arises as to whether this is also true for the reaction of enzyme with substrate. To test this possibility, the following pulse–chase experiment was performed. PMS (49 $\mu$M) was added to methanol dehydrogenase (292 $\mu$M) in 250 $\mu$L of 0.3 M sodium borate, pH 9.0/40 mM $NH_4Cl$ at 25 °C. After 10 s, 200 $\mu$L was transferred to 800 $\mu$L of rapidly stirring buffer containing [$^{14}$C]methanol (final concentration 216 $\mu$M, $10^5$ cpm/nmol). After a further 5 s the reaction was quenched by adding 20 $\mu$L of 70% (w/v) perchloric acid and transferred to ice. Ideally, only enzyme molecules that have reacted with PMS in the first incubation are able to react with [$^{14}$C]methanol upon dilution. Under the conditions used in the first incubation, the spectral change to form B occurs in under 10 s. Two control experiments were performed, one in which the PMS was added to the second incubation and one in the absence of enzyme. A second experiment was done with the following modification: The quenching mixture had a final volume of 2.5 mL, and the reaction was stopped with 50 $\mu$L of 70% (w/v) perchloric acid. The results of these experiments are shown in Table III. The number of enzyme turnovers was calculated on the assumption that 14% of the enzyme was active. Preincubation with PMS resulted in an average of 0.72 additional enzyme turnover. Thus the enzyme reacts with PMS before reacting with methanol.

The effect of preincubating the enzyme with [$^{14}$C]methanol and then diluting it into a solution containing PMS and excess

Table III: Effect of Preincubation with PMS on the Reaction of Methanol Dehydrogenase with Methanol. Pulse–Chase Experiments[a]

| | no. of enzyme turnovers | | |
|---|---|---|---|
| | enzyme preincubated with PMS | no preincubation with PMS | difference in no. of turnovers |
| first experiment | | | |
| data from HPLC | 3.37 | 2.50 | 0.87 |
| data for derivative | 3.85 | 3.18 | 0.67 |
| second experiment | | | |
| data from HPLC | 2.50 | 1.89 | 0.61 |
| data for derivative | 2.28 | 1.55 | 0.73 |

[a] For experimental conditions, see text.

unlabeled methanol was also investigated. Preincubation with methanol did not increase the amount of product formed. Therefore, the enzyme does not form a complex with methanol in the absence of PMS of sufficient lifetime to be detected in this type of experiment.

*Detection of $O_2$ Uptake during Reaction of Methanol Dehydrogenase with PMS, Cyclopropanol, and Potassium Cyanide.* The possibility was considered that the reactions of the enzyme with PMS or with PMS and cyclopropanol or potassium cyanide were redox reactions. Methanol dehydrogenase (305 $\mu$M) was incubated at 25 °C in 0.3 M sodium borate, pH 9.0/40 mM $NH_4Cl$ in the $O_2$ electrode. PMS (45 $\mu$M) followed by cyclopropanol (45 $\mu$M) was added to the chamber and the oxygen concentration monitored. After 10 min the activity of the enzyme was assayed. Two other experiments were performed with PMS and cyclopropanol, one in which the order of addition of PMS and cyclopropanol was reversed and one using only PMS. In the latter case the enzyme activity was assayed after 40 min. In each experiment the enzyme was completely inactivated and no oxygen uptake was observed. The reaction of methanol dehydrogenase with PMS and potassium cyanide (5 mM) was followed in a similar way. Again no oxygen uptake was observed, but in this case the enzyme was not inactivated. If any of the above reactions had required 1 mol of $O_2$ per mole of active enzyme (assumed to be 14% of the total enzyme), then 17 nmol of $O_2$ would have been consumed in these experiments. A minimum of 1.0 nmol of $O_2$ could have been detected.

*UV–Visible Absorption Spectrum of PMS in the Presence of Methanol Dehydrogenase.* The reaction of PMS with methanol dehydrogenase does not involve $O_2$ uptake. It is possible, however, that the PMS is reduced in the reaction and is unable to react with $O_2$. To test this possibility, the spectrum of PMS (3.4 $\mu$M) in 0.3 M sodium borate, pH 9.0/40 mM $NH_4Cl$ was recorded in the absence of the enzyme and 2 min after the addition of 24 $\mu$M enzyme. Oxidized PMS has an absorbance maximum at 387 nm (Zaugg, 1964). The absorbance at this wavelength was increased by 6% when the enzyme was added. This can be accounted for by the change in the enzyme spectrum in the presence of PMS. If PMS were reduced during the formation of the enzyme species B, a decrease in absorbance at 387 nm would have been expected.

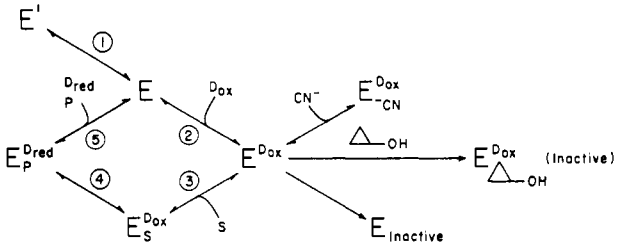
*Inactivation of Methanol Dehydrogenase by Cyclopropanol.* The characteristics of this reaction and the products formed are different from those described by Mincey et al. (1981). The preparation of methanol dehydrogenase used in these investigations requires PMS for inactivation by cyclopropanol. Several features of this reaction have been described in earlier sections. The spectrum of the enzyme is altered after reaction with cyclopropanol (Figure 1, spectrum 3). This spectral change is retained even when the excess cyclopropanol and PMS have been removed by gel filtration. Under the conditions of inactivation shown in Figure 5, the rate of inactivation shows no deuterium kinetic isotope effect. The rate of the spectral change at 400 nm is identical for [1-$^2$H]cyclopropanol and [1-$^1$H]cyclopropanol. The data in Figure 4 suggest that cyclopropanol at 12–17% of the total enzyme concentration is sufficient to completely inactivate the enzyme. This observation is confirmed by inactivating the enzyme with [1-$^{14}$C]cyclopropanol. The radioactivity that is incorporated into the protein corresponds to 0.13 ± 0.03 mol of cyclopropanol/mol of enzyme.

In order to investigate the fate of the cyclopropanol during the inactivation, the enzyme was added to a mixture of [1-$^{14}$C]cyclopropanol and [1-$^3$H]cyclopropanol. Methanol dehydrogenase (200 $\mu$M) in 1.5 mL of 0.3 M sodium borate, pH 9.0/40 mM $NH_4Cl$ was completely inactivated by incubation with [1-$^3$H,1-$^{14}$C]cyclopropanol (340 $\mu$M, $10^3$ cpm/nmol) and PMS (175 $\mu$M) for 20 min at 25 °C in the dark. The inactivation mixture was passed through a Sephadex G-25 column (40 cm × 1.5 cm diameter) in 50 mM sodium phosphate, pH 7.0. The protein fractions were pooled and precipitated with 2 volumes of methanol, and the precipitated protein was removed by centrifugation. The supernatant fluid contained 85–95% of the radioactivity from the protein pool. The supernatant fluid was taken to dryness on a rotary evaporator; less than 1% of the radioactivity was volatile. The residue was dissolved in 0.5 mL of $H_2O$ and analyzed by HPLC using the $C_{18}$ column. Peaks with the following elution times were observed: 12–15, 23, and 54 min. Of the radioactivity loaded onto the column 85–90% was found in the peak at 54 min, 5% in the peak at 12–15 min, and none in the peak at 23 min. The radioactive peaks contained both tritium and carbon-14. The peak at 12–15 min, the column breakthrough, contained some protein. The peak at 23 min has the spectrum of the normal cofactor, and the peak at 54 min has a spectrum similar to the acetone adduct of the cofactor (Duine & Frank, 1980b). The combined recovery of the cofactor and the adduct (calculated after conversion to the cofactor, see below) was 20–30% on the assumption of 1 mol of cofactor per mole of enzyme and an extinction coefficient for the cofactor of 1.8 × $10^4$ $M^{-1}$ $cm^{-1}$ at 249 nm (Duine et al., 1980a).

The adduct was found to be unstable. The main degradation pathway, which was accelerated by incubation at pH 11 but not at pH 2, was to the cofactor, releasing a radioactive molecule that eluted at 12–15 min on the $C_{18}$ column. The radioactive molecule contained both tritium and carbon-14. It was nonvolatile at neutral and acid pH. Attempts were made to identify the radioactive species by comparing its elution time on the HPLC Aminex ion-exclusion column with various standards. This was unsuccessful, however; the species is not propionaldehyde, cyclopropanol, or acrolein, nor did the solution contain any [$^3$H]$H_2O$. The above discussion describes the results generally obtained for the inactivation of the enzyme by cyclopropanol. However, in some cases, the following additional products were observed on the $C_{18}$ column: [$^3$H]$H_2O$ in the 12–15 min peak and unlabeled modified forms of the cofactor eluting at 60 min and 64 min.

Inactivation of methanol dehydrogenase using a mixture of [1-$^{14}$C]- and [1-$^3$H]cyclopropanol allows the tritium isotope effect for the inactivation reaction to be calculated. The inactivation reaction was performed in two different ways. In the first experiment the enzyme was inactivated with a 1.4-fold molar excess of cyclopropanol (assuming 13% of the enzyme was active). In this case the majority of the cyclopropanol will react, and any isotope effect will be expressed as an enrichment in tritium in the unreacted cyclopropanol. The

Scheme I




specific activities of the initial cyclopropanol mixture and of the cyclopropanol remaining after the reaction were compared. The unused cyclopropanol in the low molecular weight pool from the G-25 column was purified on $C_{18}$ HPLC before determining its specific activity. It was found to be the only radioactive species in this pool as expected. In the second experiment the enzyme was inactivated with a 50-fold molar excess of cyclopropanol. The specific activity of the initial cyclopropanol mixture was compared with the specific activities of the inactive enzyme and the subsequently isolated adduct. The data from these experiments were analyzed as described by Cleland (1982). The calculated isotope effects were 1.05 from the first experiment, 0.96 from the second experiment based on the protein-bound product, and 1.07 from the second experiment based on the isolated adduct. These results suggest that there is no tritium isotope effect for the inactivation of methanol dehydrogenase by cyclopropanol. However, the isotope ratios could not be determined sufficiently accurately to exclude a secondary tritium isotope effect.

## Discussion

The results reported here and previously (Mincey et al., 1981) lead to the conclusion that oxidation of alcohols by alcohol dehydrogenase is a two-step process. Initially, a complex between enzyme and dye is formed. This complex, then, reacts with alcohol and $O_2$ to produce aldehyde and $H_2O_2$. No evidence for the intermediate formation of reduced cofactor (methoxatin) has been obtained. It is sufficient to postulate that the enzyme catalyzes the transfer of electrons from substrate to dye and the reduced dye reacts with $O_2$. Presumably methoxatin participates in the transfer of electrons. A sequence of reactions consistent with our data is shown in Scheme I. E′ is the form of the enzyme (spectrum 1, Figure 1) that is first isolated. It is transformed to E at high pH. This reaction is accelerated by dye but does not require dye. $D_{ox}$ and $D_{red}$ represent oxidized and reduced dye, respectively. S and P are the substrate and product. According to Scheme I, initially, reaction occurs between enzyme (E) and dye to give the complex $E^{D_{ox}}$. This reaction is not a redox process. E and $E^{D_{ox}}$ are the species whose absorbance at 400 nm (Figure 2) corresponds to A and B, respectively. The enzyme–dye complex ($E^{D_{ox}}$) can undergo several reactions: (1) a reversible reaction with $CN^-$; (2) reaction with cyclopropanol to yield an inactive complex; (3) reaction with substrate through reactions 3–5 to give products; (4) transformation to a catalytically inactive species in the absence of substrate or CN.

The following supports the proposed reaction sequence. We have previously shown, and now confirmed, that an isotope effect occurs when the enzyme oxidizes $[^2H_3]CH_3OH$ at saturating concentrations. The isotope effect is maximal at high dye concentration and approaches unity at low dye concentrations. This suggests a two-step process, in which reaction with dye (step 2, Scheme I) is rate determining at low dye concentrations and reaction with $CH_3OH$ (step 4, Scheme I) is rate determining at high dye concentrations.

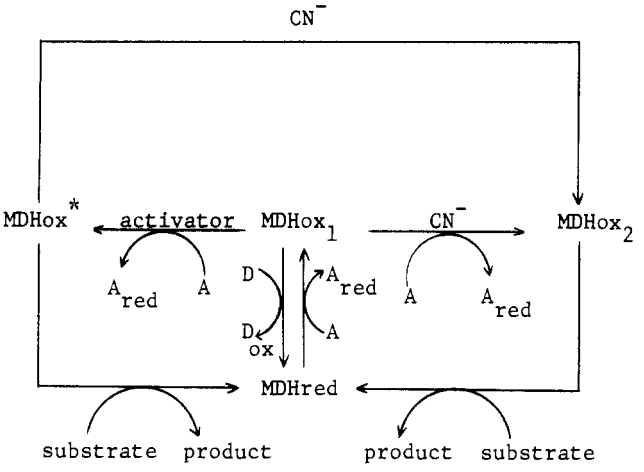
From the isotope effect alone, it cannot be determined whether reaction with dye (as shown in Scheme I) or reaction with methanol occurs first. Addition of methanol to the enzyme, in the absence of dye, produces no spectral change. In addition, we have previously observed (Mincey et al., 1981) that the enzyme, in the absence of dye, catalyzes no partial reactions; i.e., no exchange of substrate hydrogens with solvent protons is observed, nor is there exchange of $^{14}CH_2O$ with $CH_3OH$. Also, when a pulse–chase experiment was done in which $^{14}CH_3OH$ and enzyme were preincubated, no radioactive product was detected. Thus, there is no support for reaction of methanol with the enzyme alone. Evidence presented in this report shows that dye reacts with enzyme prior to addition of substrate. In the pulse–chase experiments in which enzyme was preincubated with dye and then diluted in the presence of $^{14}CH_3OH$, product was formed equivalent to ~75% of the amount of active enzyme present. The minimal conclusion is that enzyme and dye form a complex whose rate of dissociation is slower than its reaction to form product.

Spectral changes at 400 nm after addition of dye to methanol dehydrogenase were examined (Figure 2). The spectral changes observed can be correlated with the enzyme forms described in Scheme I. Upon addition of dye a species A (E, Scheme I) is formed. The complete spectrum of this species is represented by spectrum 2 of Figure 1. This transformation is represented by reaction 1 in Scheme I. Next a species designated B (Figure 2) ($E^{D_{ox}}$, Scheme I) is formed. The rate of formation of B ($E^{D_{ox}}$) is dependent on the concentration of dye and enzyme. Since the spectrum of species A is similar to that observed at steady state in the presence of substrate and low concentrations of dye, it is possible that the time period required for the conversion of A to B is due to the consumption of trace amounts of substrate. If no substrate is added, species B decays and loss of catalytic activity occurs ($E_{inactive}$, Scheme I). If $CN^-$ or cyclopropanol is added, the absorbance at B increases (Figure 4). When the loss of catalytic activity is measured after addition of cyclopropanol, a lag period of approximately 2 min is observed (Figure 5). This lag period closely corresponds to the time required for the formation of B (Figure 4). These results suggest that cyclopropanol reacts with B. This was confirmed in an experiment in which it was shown that the rate of inactivation of methanol dehydrogenase by cyclopropanol increased when the enzyme was incubated with dye prior to addition of cyclopropanol (Table II).

We suggest that species B ($E^{D_{ox}}$, Scheme I) is also the form of the enzyme that can react with alcohol to give an aldehyde. This is supported by a pulse–chase experiment, which shows that a complex is formed between enzyme and dye that can react with methanol to yield formaldehyde.

Species B ($E^{D_{ox}}$) is formed through the interaction of enzyme and dye. This could involve two types of interactions: (1) If the enzyme is present in a reduced form prior to reaction with dye, the interaction of dye and reduced enzyme could lead to oxidation of the enzyme and reduction of the dye. (2) A complex is formed between enzyme and dye that does not involve electron transfer but could involve chemical interaction. We consider the first type of interaction unlikely since reaction of enzyme and dye does not lead to $O_2$ uptake, nor does it involve a change in the spectrum of the dye. We, therefore, prefer the second of the two possibilities. A specific interaction with an artificial electron acceptor may seem surprising. However, it is consistent with the observation that only a few fairly hydrophobic electron acceptors will function with methanol dehydrogenase (Duine et al., 1978). The importance

Scheme II




of hydrophobic interactions is also suggested by the fact that methanol dehydrogenase, and the cytochrome *c* to which it is coupled, is probably loosely bound to the periplasmic side of the respiratory membrane in vivo (Jones et al., 1982).

The spectral forms of methanol dehydrogenase shown in Figure 1 have been described before by Duine and co-workers (Duine & Frank, 1981; de Beer et al., 1983). However, some of their interpretations concerning the oxidation state of various forms of the enzyme differ from our interpretations. The reaction scheme proposed by these authors is shown in Scheme II (de Beer et al., 1983).

On the basis of absorption spectra, the following correspondence between the enzyme forms in Schemes I and II exists: $MDH_{ox_1} = E'$; $MDH_{ox}* = E^{D_{ox}}$; $MDH_{ox_2} = E^{D_{ox}}_{CN}$; $MDH_{red} = E$. We are in agreement in that $MDH_{ox_1}$ (E′) can react with dye and substrate to give reaction products and the species that they designate $MDH_{red}$. However, we disagree concerning the oxidation states of the various enzyme forms. For reasons cited above, we do not believe that the conversion of E′ to E ($MDH_{ox_1} \rightarrow MDH_{red}$) involves electron transfer; i.e., the designation $MDH_{red}$ is inappropriate. Nor do we believe that the formation of the $CN^-$ adduct ($MDH_{ox_1} \rightarrow MDH_{ox_2}$) involves electron transfer. According to our interpretation, that reaction involves reversible addition of $CN^-$ to $E^{D_{ox}}$ (Scheme I). The basic difference derives from the fact that we have no evidence that reaction of enzyme and dye, in the absence of substrate, leads to dye reduction; i.e., there is no $O_2$ uptake, and the dye does not undergo a spectral change. It must be pointed out that we are comparing experimental results obtained with enzymes derived from different sources. However, we do not believe that they operate by different mechanisms.

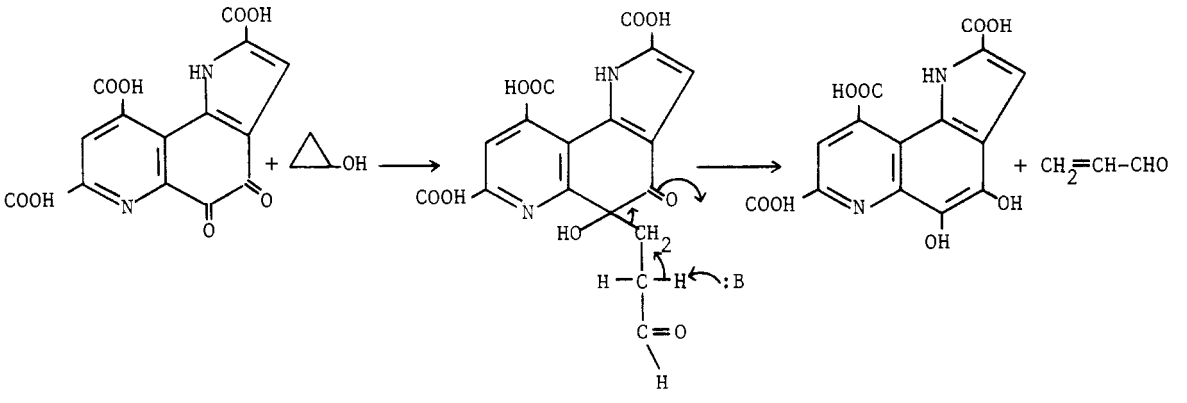
We have previously reported that approximately 10–15% of our enzyme preparation is catalytically active, although, on the basis of spectral data, at least/approximately 70% of the enzyme molecules contain cofactor. The low percentage of catalytically active enzyme is confirmed by results reported here. In the reaction with [1-$^{14}$C]cyclopropanol the incorporation of 0.13 mol of [1-$^{14}$C]cyclopropanol per mole of enzyme is sufficient to completely inactivate the enzyme. Similarly, the spectral change observed in the presence of cyclopropanol is complete in the presence of 0.12–0.17 mol of cyclopropanol per mole of enzyme.

The conclusion that approximately 13% of the enzyme is catalytically active is difficult to reconcile with data in Figures 1 and 2. Addition of substoichiometric amounts of dye causes a decrease in absorbance of more than 13% at 340 nm. A possible explanation is that all of the enzyme molecules can react with dye although not all molecules are catalytically active and that the dye acts catalytically.

Reaction of enzyme and cyclopropanol results in the formation of an adduct between cyclopropanol and methoxatin. The spectrum of the adduct is similar to that formed with $CN^-$ (Figure 1), suggesting a similar reaction mode. The spectrum of the isolated (HPLC) adduct is very similar to the adduct found between acetone and methoxatin (Duine & Frank, 1980b), which suggests that an adduct is formed with one of the carbonyl groups of methoxatin. The adduct formed with cyclopropanol decomposes under basic conditions to regenerate methoxatin and as yet unidentified molecules derived from cyclopropanol. Insufficient information is available to define the structure of the methoxatin–cyclopropanol adduct. In Scheme III a possible structure and its mode of decomposition are shown. The adduct could be formed either through an ionic or through a radical process. At this time we have no evidence that allows us to decide which mechanism applies. Our failure to isolate acrolein could be due to the reactivity of this compound, i.e., its tendency to react with nucleophiles.

The enzyme preparation used in these experiments differs in some respects from that previously used. It was previously reported that addition of cyclopropanol to alcohol dehydrogenase in the absence of dye led to loss of catalytic activity. With the enzyme preparation used in these experiments no inactivation occurs in the absence of dye. Previously it was reported that the enzyme gave an ESR signal, the concentration of which was equivalent to approximately 15% of the enzyme present. Others have also observed similar ESR spectra. This has led to the conclusion that a methoxatin-derived radical is important in catalysis and that the active form of the enzyme contains a methoxatin semiquinone (Mincey et al., 1981). Clearly, these results show that the catalytically active enzyme need not be in the semiquinone

Scheme III



form. The preparations of methanol dehydrogenase used in this investigation showed a free-radical signal corresponding to only 1% of the total enzyme. The reason for these discrepancies is not obvious. Possibly, the preparation previously used was contaminated with an endogenous electron acceptor. It must be pointed out that the lack of a radical signal in the enzyme preparation used in these experiments does not rule out the intermediate participation of a methoxatin radical in catalysis. It is possible, for instance, that a radical is generated in the presence of dye and substrate.

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# Physical and Enzymatic Properties of a Class II Alcohol Dehydrogenase Isozyme of Human Liver: π-ADH†

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ABSTRACT: Homogeneous class II alcohol dehydrogenase (π-ADH) has been isolated from human liver homogenates by chromatography on DE-52 cellulose, 4-[3-[*N*-(6-aminocaproyl)amino]propyl]pyrazole–Sepharose, SP-Sephadex C-50, and agarose–hexane–AMP, yielding an enzyme that has a significantly higher specific activity and is markedly more stable than that isolated by an earlier procedure. π-ADH is composed of two identical 40 000-dalton subunits, contains 4 mol of zinc/dimer, and is readily inhibited by metal-chelating agents. The purified enzyme binds two molecules of coenzyme per dimer, exhibits an absorption maximum at 280 nm, $\epsilon_{280}$ = 57 000, and exhibits an isoelectric point of 8.6. The class II isozyme catalyzes the oxidation of a variety of alcohols with $K_m$ values ranging from 7 μM to 560 mM and with $k_{cat}$ values from 32 $min^{-1}$ to 600 $min^{-1}$ and demonstrates a preference for hydrophobic substrates. The $k_{cat}/K_m$ ratio for ethanol oxidation exhibits a pH maximum at 10.4.

Alcohol dehydrogenase (ADH)[1] (alcohol:$NAD^+$ oxidoreductase, EC 1.1.1.1) is the primary enzyme responsible for the oxidation of ethanol, the first step of ethanol elimination in humans (Li, 1977). This enzyme represents 2–3% of the soluble protein in normal human liver, which suggests that, aside from its action on exogenous ethanol, it may serve an important though presently unknown function(s) in intermediary metabolism. Efforts to elucidate the metabolic role of liver ADH have been frustrated by the diversity of its mammalian isozymes (Brandén et al., 1975). Thus, more than 20 have now been identified in human liver (von Wartburg et al., 1965; Blair & Vallee, 1966; Smith, et al., 1971; Bosron et al., 1979a, 1980; Parés & Vallee, 1981; Wagner et al., 1984).

The ADH isozyme makeup of human liver is apparently a function of the genetic background and state of health of the individual (Smith et al., 1973; Li & Magnes, 1975; Ricciardi et al., 1983). These isozymes have been differentiated into three classes on the basis of their electrophoretic mobilities and inhibition by 4-methylpyrazole (Strydom & Vallee, 1982;

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[1] Abbreviations: ADH, alcohol dehydrogenase; CapGapp, 4-[3-[*N*-(6-aminocaproyl)amino]propyl]pyrazole; HPLC, high-performance liquid chromatography; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; SDS, sodium dodecyl sulfate.

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