Mechanism of Action of Methoxatin-Dependent Alcohol Dehydrogenase†

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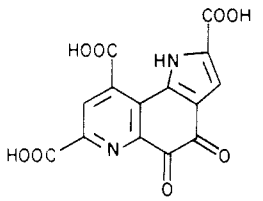
ABSTRACT: Methoxatin (I) is a cofactor associated stoichiometrically with bacterial methanol dehydrogenase. From the electron spin resonance (ESR) spectrum it was determined that approximately 13% of the cofactor is present in the semiquinone form. Inactivation of the enzyme leads to concomitant loss of the semiquinone and enzyme activity. Addition of substrate to the enzyme produces no detectable change in the absorption spectrum of the cofactor nor in the radical concentration. Oxidation of $[^2H]CH_3OH$ shows an isotope effect of 4.6 in the presence of high phenazine methosulfate (PMS; electron acceptor) concentration and essentially no isotope effect at low PMS concentration. This result is consistent with the formation of reduced enzyme and subsequent reoxidation of the reduced enzyme by PMS. At high PMS concentrations, oxidation of methanol is at least partially rate determining. At low PMS concentrations reoxidation of the reduced enzyme becomes rate determining. When [1-^{3}H]butanol is added to the enzyme in the absence of PMS, no $[^3H]H_2O$ is produced. Addition of propionaldehyde and [1-^{3}H]butanol did not result in $[^3H]H_2O$ formation or in the formation of [1-^{3}H]propanol. Cyclopropanol irreversibly inactivates the enzyme in the absence of PMS. Cyclopropanol stoichiometrically equivalent to 14% of the enzyme completely inactivates the enzyme. The inactivation shows an isotope effect of 4 when [1-^{2}H]cyclopropanol is used. When [1-^{3}H]cyclopropanol is used, no $[^3H]H_2O$ is formed. Approximately 60–70% of the radioactivity is covalently bound to the enzyme, and 30–40% is released as an as yet unidentified small molecule. It is proposed that cyclopropanol is oxidized by the enzyme and reaction of the enzyme with oxidation product leads to inactivation. We conclude that only semiquinone forms of the cofactor are catalytically active. Addition of substrate to the enzyme leads to a two-electron reduction of the semiquinone form of the enzyme. The lack of isotope exchange suggests that the substrate hydrogen is transferred to the enzyme when the substrate is oxidized and is not subject to exchange with solvent protons and the oxidation product is probably not released until the enzyme is reoxidized by PMS.

A number of bacterial dehydrogenases have been described that in vitro oxidize primary alcohols to aldehydes in the presence of the electron acceptor phenazine methosulfate (PMS) and the activator NH_4^+ (eq 1).

$$RCH_2OH \xrightarrow[PMS_{red}]{PMS_{ox}} RCHO \qquad (1)$$

Methoxatin (I), a cofactor whose structure has been recently



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established by X-ray analysis (Salisbury et al., 1979) of a derivative and by chemical characterization (Duine et al., 1980; Duine & Frank, 1980a), is tightly bound to these enzymes. These alcohol dehydrogenases also contain a stable free radical that is believed to be due to the semiquinone form of methoxatin (Westerling et al., 1979; de Beer et al., 1979; Duine et al., 1978). The electron spin resonance (ESR) and external nuclear double resonance (ENDOR) spectra of the semiquinone of methoxatin free in solution as well as the enzyme-bound forms have been studied (Westerling et al., 1979; de Beer et al., 1979). These studies have also aided the proof of structure of the cofactor. It is therefore apparent that the enzyme-bound cofactor must be present in at least two oxidation states, the quinone and semiquinone.

It is interesting that other methanol-oxidizing enzymes, which are flavoprotein oxidases, also contain a stable free radical, a flavin semiquinone, and oxidized flavin (Mincey et al., 1980). It is known that the enzyme isolated from *Hansenula polymorpha* does not require the flavin semiquinone for the oxidation of methanol in vitro (Mincey et al., 1980). A reconstituted enzyme that contains oxidized flavin is nearly fully active in catalysis.

Nothing is known, at present, concerning the role of the two oxidation states of methoxatin in catalysis or, for that matter, whether any form of methoxatin participates in catalysis. Thus, no change in the optical spectrum of enzyme-bound methoxatin occurs when substrate is added to the enzyme (Duine et al., 1978). Enzyme from which methoxatin has been removed is catalytically inactive (Patel et al., 1978). However, so far attempts at reconstitution of the pure enzyme have not succeeded. It has been claimed that addition of methoxatin to methoxatin-dependent glucose oxidase in unfractionated extracts enhances catalytic activity (Duine et al., 1979). However, we found these experiments difficult to analyze. Despite the lack of clear-cut evidence as to the role of the cofactor, several proposals have been made for the role of methoxatin. It has been suggested that the quinone form of methoxatin serves as a two-electron acceptor in the oxidation of methanol (Duine & Frank, 1980b). A mechanism has also been proposed involving the nucleophilic addition of the alcohol substrates to the carbonyl group of methoxatin. Subsequent

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proton abstraction from C-1 of the alcohol and electron transfer to methoxatin result in oxidation of the alcohol (Forrest et al., 1980). A similar mechanism has been proposed for the oxidation involving flavins (Hamilton, 1973) and has been criticized on chemical grounds (Bruice, 1980). Again, this mechanism has no experimental support.

To learn more about the role and mechanism of action of methoxatin, we have investigated a methanol dehydrogenase from *Methylomonas methanica* (Patel et al., 1978). This enzyme has been obtained in crystalline form, and some of its properties and substrate specificity have been described (Patel et al., 1978). The enzyme has a molecular weight (M_r) of 60000 and is a monomer. In this respect, it differs from other methanol dehydrogenases that are dimers with a subunit M_r of 60000 (Duine et al., 1979). In all other respects, it appears to be very similar to the other methanol dehydrogenases.

Materials and Methods

Growth of Microorganisms. M. methanica strain S1 was the gift of Dr. R. W. Patel. The organism was maintained on agar plates containing a mineral salts medium and methanol, 0.4% v/v, as the sole carbon source (Patel et al., 1978; Foster & Davis, 1966). The organism was grown in 100 L of medium in a Chemap A.G. fermentor with maximal aeration at 30 °C in a mineral salts medium that contained 0.4% v/v methanol as the sole carbon source (Patel et al., 1978; Foster & Davis, 1966). The cells were harvested in late log phase by a continuous-flow centrifuge, were frozen, and were stored at −20 °C prior to use.

Enzyme Purification and Assay. The frozen bacteria were thawed at 4 °C, and the enzyme was then purified (Patel et al., 1978). The enzyme was routinely stored in 50% glycerol and 50% sodium phosphate (0.05 M; pH 7.0) containing 10 mM methanol at −80 °C.

Before use the enzyme preparations, stabilized by glycerol, were thawed at 5 °C and dialyzed extensively against 0.05 M sodium phosphate buffer, pH 7.0, to remove all glycerol and methanol from the enzyme. The protein in the dialyzed enzyme solution was concentrated to 15 mg/mL by use of a Millipore LX-10 ultrafiltration device. One volume of this concentrated protein solution was diluted with 10 volumes of 0.1–0.3 M sodium borate or 0.1 M tris(hydroxymethyl)-aminomethane (Tris) buffer, pH 9.0, and reconcentrated to the desired protein concentration.

The enzyme was assayed in an oxygen electrode by observing the reduction of oxygen or with a spectrophotometric assay (Patel et al., 1978, 1972). The oxygen-electrode assay was performed at 25 °C in a thermostated cell in a total volume of 0.4 mL. The rate of oxygen reduction was displayed on a recorder, and initial rates were obtained from this trace. Each assay contained 0.3 M sodium borate buffer, pH 9.0, 40 mM ammonium chloride, 8 mM methanol, and 5 mM phenazine methosulfate and was initiated by the addition of enzyme. One unit of enzyme is defined as the amount required to reduce 1 μmol of oxygen to hydrogen peroxide per min at 25 °C.

Spectrophotometric assays were carried out in solution with the composition 0.25 M sodium borate buffer, pH 9, 35 mM NH_4Cl, 3 mM CH_3OH, 6 mM PMS, and 3 mM dichlorophenolindophenol (DCIP). The reaction is begun by adding enzyme to this solution in a cuvette and mixing quickly by inverting several times. The absorbance at 700 nm is recorded, and the rate is determined from the slope of the trace between 20 and 30 s after enzyme addition. The extinction coefficient of DCIP is 5.2×10^3 M^{-1} cm^{-1} at 700 nm (calculated from the spectrum and extinction coefficient of 2.2×10^4 M^{-1} cm^{-1} at 600 nm) (Armstrong, 1964). Assays that give rates of greater than 80 nmol of DCIP mL^{-1} min^{-1} correspond closely to the rates observed in the O_2-electrode assays on the assumption that 1 mol of O_2 is stoichiometrically equivalent to 1 mol of DCIP as the ultimate two-electron acceptor. However, under these assay conditions the reoxidation of DCIP by dissolved O_2 occurs. We have corrected this reoxidation by adding 8 nmol mL^{-1} min^{-1} to the observed oxidation rate. All rates and, hence, enzyme activities determined spectrophotometrically were corrected by this factor. Protein was determined by the method of Lowry et al. (1951).

Scintillation Counting. Samples were dissolved in 10.0 mL of aqueous counting scintillant (ACS; Amersham) and counted on a Beckman LS-100 C liquid scintillation system. The counting efficiency was determined by addition of a radioactive tritium standard (New England Nuclear) to the counted sample.

Absorption Spectra. All absorption spectra were obtained on a Perkin-Elmer Model 559 spectrophotometer with 1-cm quartz cells at 25 °C.

Electron Spin Resonance Spectra. Electron spin resonance spectra were obtained on a Varian E-4 or electron spin resonance spectrometer at −169 °C and a Varian E-7 EPR spectrophotometer at 25 °C. Standard experimental parameters were as follows: time constant 1 s; modulation amplitude 2 G; receiver gain 5×10^3; microwave power 2 mW; field set 3240 G; microwave frequency 9.148 GHz.

Cofactor Isolation from the Enzyme. To the enzyme, 4.9 mg (82 nmol) in 1.0 mL of 0.3 M Tris buffer, pH 9.0, was added 9.0 mL of methanol, and the precipitated protein was centrifuged at 10000*g* for 15 min. The supernatant methanol containing the cofactor was removed and the protein resuspended in 5 mL of fresh methanol. The protein was centrifuged again at 10000*g* for 15 min and a second methanol fraction obtained. The methanol fractions were combined and the methanol was removed at reduced pressure. The residue was dissolved in 0.5 mL of 0.1 M sodium borate buffer, pH 9.0, and the absorption spectrum recorded.

Chromatographic Separation of Alcohols and Water. In isotope-exchange experiments it was necessary to separate water from volatile alcohols. This was done as follows: The reaction mixture was bulb-to-bulb distilled at reduced pressure. The distillate was then subjected to high-pressure liquid chromatography (HPLC) (μBondapak C_{18} column, 1.4 × 27 cm). The column was eluted with water at a flow rate of 1.0 mL/min. The following retention volumes (expressed as multiples of the void volume) were obtained: H_2O, 1.0; ethanol, 2.0; 1-butanol, 6.4. The ability to recover ^3H_2O was established by injecting ^3H_2O into the column. The overall recovery of ^3H_2O was 70%.

Chemicals. Cyclopropyl methyl ketone and 18-crown-6 were obtained from Aldrich, phenazine methosulfate (PMS) was from Sigma, and methanol and formaldehyde were reagent grade from Fisher. ($^2H_4$)Methanol was a gift of Dr. A. Redfield, Brandeis University. ^2H_2O was obtained from Bio-Rad, ^3H_2O and [^{3}H]$NaBH_4$ were from New England Nuclear, and ACS for scintillation counting was from Amersham.

Syntheses. 1-[1-^{3}H]Butanol. To 1.92 mg (0.06 mmol) of sodium [^{3}H]borohydride (25 mCi, 400 mCi/mmol) in 0.1 mL of water was added 28 mg (0.34 mmol) of 1-butanal. After the solution was maintained for 20 min at 25 °C, 16 mg (0.16 mmol) of sulfuric acid, 140 mg (1.0 mmol) of semicarbazide hydrochloride, 1.0 mL of water, and 0.5 mmol of 1-butanol were added. The volatile components were distilled by bulb-

to-bulb distillation at reduced pressure.

The 1-[1-^{3}H]butanol was purified by injection of the bulb-to-bulb distillate on a μBondapak C_{18} HPLC column, 1.4 × 27 cm (Waters Associates, Mildford, MA), and elution with pure water. The 1-butanol peak was detected at 6.4 void volumes by use of a refractive index detector. The aqueous butanol fractions were combined. The concentration of butanol was determined by HPLC by comparison to peak areas of butanol solutions of known concentrations.

Cyclopropanol. Cyclopropanol was prepared from cyclopropyl methyl ketone by the previously described method (DePuy & Mahoney, 1964). The crude cyclopropanol was dissolved in water and purified by preparative HPLC with a μBondapak C_{18} column. Pure water was used as the eluant.

The aqueous fractions that eluted at 3.0 void volumes were collected and combined. To this solution enough solid sodium chloride was added to yield a saturated solution. The cyclopropanol was extracted from this solution with ether and the cyclopropanol reextracted from the ether by a small volume of ^2H_2O. HPLC of an aliquot of this cyclopropanol solution in ^2H_2O showed a single peak at 3.0 void volumes. The proton nuclear magnetic resonance (^{1}H NMR) spectrum of the cyclopropanol in ^2H_2O displayed proton resonances at δ 3.4 (multiplet) and δ 0.45 (doublet). An infrared spectrum of the cyclopropanol, obtained by careful evaporation of the ether solution, displayed peaks at 3400, 3100, 3010, and 1015 cm^{-1}. These properties are identical with the literature values previously reported (Martinez et al., 1975).

The concentration of the cyclopropanol in ^2H_2O was determined by addition of a known amount of 18-crown-6 to the ^2H_2O solution and a comparison of the integrals of the proton resonances of the cyclopropanol and of the crown ether peak at δ 3.7 (singlet). The use of the internal standard was found to be reproducible and accurate in the determination of concentrations of solutes to ±7% when the concentrations of ethanol in standard solutions were determined in this manner. The ^2H_2O solutions of cyclopropanol were then employed to inactivate the enzyme.

[1-^{2}H]Cyclopropanol. This compound was prepared as described above from the deuterated ketone, [1-^{2}H]cyclopropyl [$^2H_3$]methyl ketone. The deuterated ketone was prepared from cyclopropyl ketone by exchange of the acidic protons with ^2H_2O in 4 N sodium deuterioxide in ^2H_2O. The basic saturated solution of the ketone was heated to 95 °C for 2 days to give complete exchange. The [1-^{2}H]cyclopropanol so obtained and purified as described above displayed a single resonance in ^{1}H NMR at δ 0.45 (singlet) (^2H_2O).

[1-^{3}H]Cyclopropanol. This compound was prepared from [1-^{3}H]cyclopropyl [$^3H_3$]methyl ketone. The tritiated ketone was prepared by the same procedure used for the preparation of the deuterated ketone except that ^2H_2O was replaced by [^{3}H]H_2O (1 Ci/mL).

The compound was purified as previously described, and chromatography of the purified material by HPLC gave a single peak at 3.0 void volumes containing 100% of the tritium. The [1-^{3}H]cyclopropanol displayed a typical NMR spectrum of cyclopropanol, δ 3.4 (multiplet) and δ 0.45 (doublet) in ^2H_2O. The specific activity of the ketone, 2.55 × 10^5 cpm/μmol, was determined by liquid scintillation counting.

Results

Properties of Methanol Dehydrogenase. The purity and specific activity of methanol dehydrogenase purified from *M. methanica* were examined (Patel et al., 1978). The pure enzyme displays a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Raymond, 1962). The specific activity obtained in the oxygen-electrode assay, with PMS, was 1.6 IU/mg. This activity is 2.5-fold greater than that reported for the crystalline preparation obtained earlier (Patel et al., 1978). The pure enzyme lost all activity after prolonged storage but could be stabilized by addition of 1 volume of glycerol to 1 volume of enzyme (10 mg/L in 0.05 M sodium phosphate buffer, pH 7.0, containing 10 mM methanol). Upon storage at −80 °C in the presence of glycerol, the color of the enzyme changes from yellow to red, and the specific activity decreases to 1.0 IU/mg. The time required for these color changes is variable. Sometimes, even fresh enzyme preparations consist predominantly of the red form. For removal of glycerol and methanol prior to use, the enzyme was extensively dialyzed at 5 °C against 0.05 M sodium phosphate buffer, pH 7.0. The enzyme is most stable in this buffer. Although the enzyme is most stable in the sodium phosphate buffer, it was not routinely possible to assay and manipulate the enzyme in this buffer since assays and many experiments were carried out at pH 9.0 where the enzyme activity is maximal (Patel et al., 1978). Therefore, other substances that buffer in the region about pH 9.0 must be employed. Previously, both Tris and pyrophosphate buffers had been employed for this purpose (Patel et al., 1978; Foster & Davis, 1966; Anthony & Zatman, 1967). However, the red enzyme preparation frequently denaturated if placed in either of these buffers. It was found that sodium borate buffer, 0.1–0.3 M, pH 9.0, greatly enhances the stability of the enzyme. Both the yellow and red enzymes can be routinely handled without loss of activity in borate buffer. Reaction rates were similar to those obtained in Tris buffer at the same pH. Therefore, it is preferable to employ sodium borate for enzyme assays and experiments if possible.

UV and Electron Spin Resonance Spectra. The absorption spectrum of the yellow enzyme, obtained immediately after purification, is shown in Figure 1. Absorbances other than those due to protein are due to the bound cofactor, methoxatin. The OD/mg of protein at 280 nm is 0.98. This value is very similar to the other methanol dehydrogenases that contain methoxatin (Duine et al., 1978; Patel et al., 1978). Likewise, the ratio of the protein absorbance at 280 nm to the cofactor absorbance at 340 nm is 10 and compares well to previous preparations (Duine et al., 1978; Patel et al., 1978) that had ratios of 9.6–10. Therefore, all the enzyme preparations contain a similar ratio of protein to cofactor. The absorption spectrum of the red form of the enzyme is shown in Figure 2. The yellow form has a specific activity of 1.6 IU/mg and the red form 1.0 IU/mg.

The spectral changes of the cofactor absorption spectrum appear to be protein induced and are not observed in the absorption spectrum of the cofactor isolated from the red form. The cofactor was resolved from 81 nmol of the red form of the enzyme [based on OD at 280 nm and protein determination (Lowry et al., 1951)] by addition to the enzyme solution of 9 volumes of methanol (Duine et al., 1978). Under these conditions, at least 68 nmol of cofactor was obtained. The concentration of cofactor was estimated from a published extinction coefficient (Duine et al., 1980). The resolved cofactor has the absorption spectrum shown in Figure 2. This spectrum matches exactly the previously reported spectrum of the oxidized form of the cofactor isolated from the yellow form of a similar enzyme (Duine et al., 1980, 1978; Duine & Frank, 1980a), and the spectrum is nearly identical with the absorption spectrum of the yellow enzyme shown in Figure 1. Therefore, the change of the spectrum in the holoenzyme is not observed in the spectrum of the isolated cofactor.

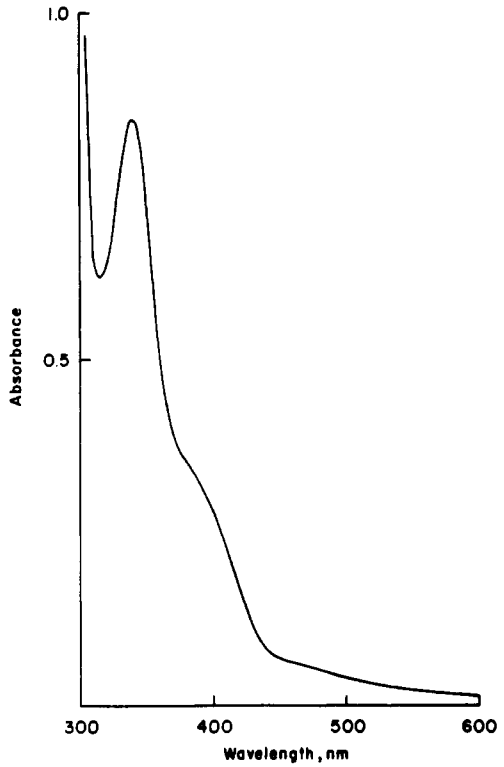

FIGURE 1: Absorption spectrum of the yellow form of the enzyme, sp act. 1.6 IU/mg, 0.133 mM in 0.1 M sodium borate buffer, pH 9.0, at 25 °C.

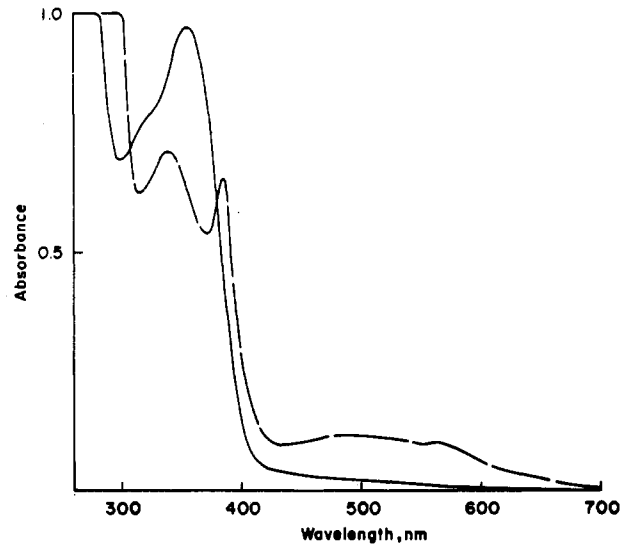

FIGURE 2: Spectra of the red form of the enzyme and the cofactor resolved from the enzyme. Spectrum of the red form of the enzyme, sp act. 1.0 IU/mg, 0.083 μM in 0.1 M sodium borate buffer, pH 9.0, at 25 °C (---); spectrum of the cofactor resolved from the red form of the enzyme, 100 μM in 0.1 M sodium borate buffer, pH 9.0, at 25 °C (—).

We find that, in our hands, the spectrum of the enzyme-bound coenzyme varies in an unpredictable way. At times, a change from phosphate buffer, pH 7.0, to borate buffer or Tris buffer at pH 9.0 causes marked spectral changes without concomitant changes in specific activity. Although the reason for these spectral changes is unknown, it is apparent that the spectrum of the enzyme is not closely related to its specific activity.

To determine if the enzyme-bound cofactor reacts with methanol in the absence of the dye PMS, we examined the visible absorption spectrum and the electron spin resonance spectrum of the enzyme. The visible absorption spectrum

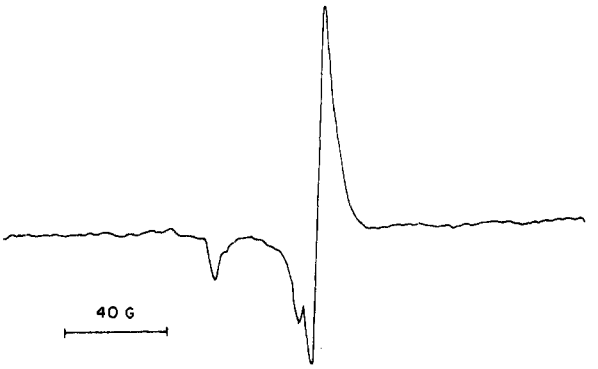

FIGURE 3: Electron spin resonance spectrum of the enzyme. The red form of the enzyme, sp act. 1.0 IU/mg, 300 μM in 0.125 M Tris buffer, pH 9.0, was placed in an EPR tube and frozen, and the spectrum shown was obtained at −169 °C.

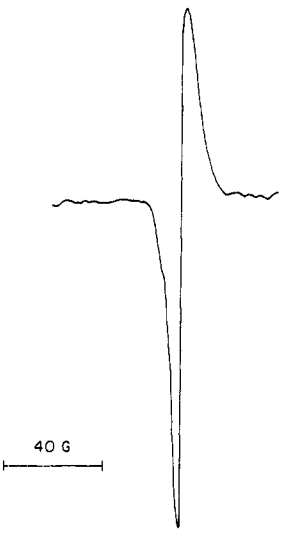

FIGURE 4: Effect of NH_4^+ in the electron spin resonance spectrum of the enzyme. To the red form of the enzyme, sp act. 1.0 IU/mg, 300 μM in 0.125 M Tris buffer, pH 9.0, at 25 °C, was added ammonium chloride, 7.3 mM. The sample was frozen and the spectrum was obtained at −169 °C.

arises from one oxidation stage of the cofactor, and the electron spin resonance spectrum arises from the semiquinone form of the cofactor. First, it was found that the visible absorption spectra of the enzyme, shown in Figures 1 and 2, are not affected by addition of either ammonium ion or methanol or both together. However, the electron spin resonance spectrum of the red form of the enzyme is changed by ammonium ion and methanol. The EPR spectrum of the enzyme in the absence of ammonium ion or methanol is shown in Figure 3. This has principle g values of 2.0045 ± 0.0003 for the main peak, 2.0100 ± 0.0003 for the shoulder, and 2.0309 ± 0.0003 for the smaller peak. The spectrum has a total width of 70 G. This signal was quantitated by comparison of the double integral of the signal to the double integral of the signal of a Cu^{2+}–ethylenediaminetetraacetic acid (EDTA) standard solution. The radical concentration was found to be 37 μM. Thus, the radical concentration is 13% of the total enzyme present. It was previously shown that the radical signal is due to the semiquinone form of the cofactor although no previous quantitation of the signal has been reported (Duine et al., 1978; Westerling et al., 1979; de Beer et al., 1979). The addition of ammonium ion to the enzyme sharpens the signal as shown in Figure 4. This peak has the same integrated intensity as the signal before ammonium ion addition and has a g value of 2.0056 ± 0.00083, a peak to peak width of 7.0 G, and a total width of 35.0 G. The addition of methanol to the enzyme

in the presence of ammonium ion has no effect on the spectrum shown in Figure 4. Further, if only methanol is added to the enzyme, with the spectrum shown in Figure 3, a spectrum identical with that in Figure 4 is obtained. The further addition of NH_4^+ has no effect on this spectrum. Thus, no change of integrated intensity of the radical signal is observed (±5%) upon the addition of substrate. However, the shape of the signal is sensitive to the activator, ammonium ion, or substrate.

The electron spin resonance spectra establish that the paramagnetic enzyme species interacts with substrate and NH_4^+. The question then arises whether the paramagnetic species is required for the catalytic activity of the enzyme. In order to determine whether upon denaturation of the enzyme, the electron spin resonance signal and catalytic activity are lost at the same rate, we slowly heat denatured the enzyme and determined the change in intensity of the electron spin resonance signal and in catalytic activity. The enzyme (sp act. 1.0 IU/mg, red form), 270 μM in 0.3 M sodium borate, pH 9.0, in a total volume of 1.0 mL, was placed in an EPR cell. The electron spin resonance spectrum was obtained at 25 °C on a Varian E-7 EPR spectrometer. The enzyme was also assayed spectrophotometrically. The EPR tube containing the enzyme was then warmed to 58 °C. At various times, the tube was cooled at 25 °C, the electron spin resonance spectrum was determined, and aliquots were removed for the spectrophotometric assay. The loss in catalytic activity closely paralleled the decrease in the intensity of the electron spin resonance signal. In another experiment the red form of the enzyme, sp act. 1.0 IU/mg, 266 μM in 0.3 M borate buffer, pH 9.0, containing 40 mM NH_4^+, was exposed to 3 mM PMS for 1 min. Exposure of the enzyme to PMS in the absence of substrate leads to loss of catalytic activity (Duine & Frank, 1980b). The enzyme was freed from PMS by passage through a P-10 column (1 × 20 cm) equilibrated with borate buffer. The intensity of the electron spin resonance signal and the catalytic activity of the enzyme were determined. Both were reduced to 10% of the values determined for enzyme that had not been exposed to PMS before chromatography. These experiments suggest that the paramagnetic species is required for catalytic activity, but this requirement is not conclusively established.[1] The visible absorption spectrum of the enzyme, inactivated by PMS, was not changed.

Inactivation of Cyclopropanol. Incubation of the enzyme with cyclopropanol results in loss of activity. A typical experiment was carried out as follows: Enzyme (sp act. 1.0 IU/mg, red form), 0.012 μM in 0.12 M Tris-HCl buffer, 10 μM NH_4Cl, and 5 μM cyclopropanol in a total volume of 100 μL, was incubated at 5.6 °C. Aliquots were periodically removed for assay of enzyme activity. Loss of enzyme activity was a first-order process with $t_{1/2}$ = 50 min. When NH_4^+ was omitted, approximately 10% of the activity was lost after 150 min. In the absence of cyclopropanol and in the presence of NH_4^+, less than 5% of the activity is lost after 180 min. The inactivation by cyclopropanol requires NH_4^+ ion as does the normal catalytic process. The rate of activity loss is proportional to cyclopropanol concentration and is reduced by the presence of methanol. When inactivated enzyme is dialyzed at 5 °C against several changes of buffer (0.05 M sodium pyrophosphate, pH 9.0), no activity is recovered. A control sample of enzyme retains 85% of its activity under the same conditions. The inactivation is therefore irreversible.

Substitution of a deuterium at C-1 of cyclopropanol lowers the rate of inactivation. Aliquots of the same enzyme solution were incubated with [1-^{2}H]cyclopropanol or the nonisotopic cyclopropanol at inhibitor concentrations that varied between 1.6 and 3.0 mM. The second-order rate constant for inactivation in each case was determined. The rate constant for inactivation by the [1-^{2}H]cyclopropanol was found to be 3-fold lower than the rate constant for inactivation by the nonisotopic compound.[2] These results indicate that the inactivation of methanol dehydrogenase by cyclopropanol involves cleavage of the C-1 carbon–hydrogen bond.

The inactivation rate shows the same buffer and pH dependence as does the rate of methanol oxidation measured in the assay with PMS.

No oxygen uptake is observed during or after inactivation of the enzyme with cyclopropanol. When 1 μmol of cyclopropanol was added to 83 nmol of enzyme and 0.4 mL of Tris, pH 9.0, containing 16 mM NH_4^+, no oxygen uptake was observed. Complete inactivation was attained. Addition of 0.4 μmol of PMS subsequent to inactivation caused no observable oxygen uptake. Less than 0.5 nmol of oxygen uptake could have been observed in these experiments. Thus, cyclopropanol inactivation does not require an additional electron acceptor.

The stoichiometry of inactivation by cyclopropanol was determined. The enzyme, 910 nmol in 0.3 M borate buffer, pH 9.0, at 25 °C containing 40 mM ammonium ion, was titrated with a standard cyclopropanol solution (9.6 nmol/μL).

The titration was carried out as follows: An amount of cyclopropanol corresponding to a fraction of the total amount of enzyme present was added, and the enzyme activity was monitored until no further change occurred. The time required for this process was approximately 2 h. This was repeated with increasing concentrations of cyclopropanol. In all cases, corrections were made for loss of activity in the absence of cyclopropanol. This activity loss was always less than 5%. From these data, the amount of cyclopropanol required for complete inactivation could be estimated. A total of 127 nmol of cyclopropanol was required to fully inactivate the enzyme, i.e., 14% of the amount of enzyme present. Duplicate runs agreed to within 10%. The amount of cyclopropanol required to inactivate the enzyme corresponds approximately to the amount of radical present.

The addition of cyclopropanol to the enzyme had no reproducible effect on the visible absorption spectrum of the enzyme. Sometimes, a 10% decrease in the 340-nm peak was observed following inactivation, and, other times, little change was observed. These changes are reminiscent of the buffer-induced changes of the spectrum, and the interpretation is unclear.

The absorption spectrum of the isolated cofactor was unchanged following inactivation. When the enzyme (81 nmol, based on OD at 280 nm) was inactivated by cyclopropanol and the cofactor resolved from the enzyme by methanol precipitation, 68 nmol or 84% of cofactor (based on the extinction coefficient) was obtained (i.e., 16% of the cofactor was not recovered). The absorption spectrum was identical with that of the cofactor spectrum in Figure 2.

Addition of cyclopropanol to the enzyme after NH_4^+ ion causes no change in the electron spin resonance spectrum, i.e., the spectrum is that shown in Figure 4. Addition of cyclo-

[1] A recent enzyme preparation (yellow form) had a specific activity of 2.0 IU/mg. The intensity of the EPR signal of this preparation was 4 times that of the red form of the enzyme with a specific activity of 1.0 IU/mg. The shape of the room-temperature spectra was very similar to that shown in Figure 4.

[2] [1-^{2}H]Cyclopropanol and the nonisotopic cyclopropanol were synthesized and manipulated in exactly the same way.

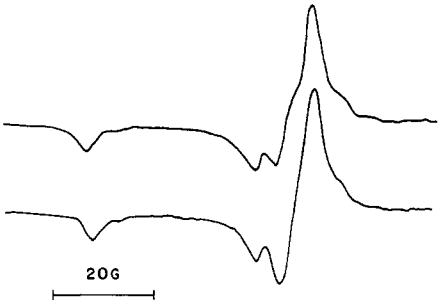

FIGURE 5: Effect of cyclopropanol on the electron spin resonance spectrum of the dehydrogenase. The red form of the enzyme, sp act. 1.0 IU/mg, 0.3 mM in 0.125 M Tris buffer, pH 9.0, was frozen and the electron spin resonance spectrum obtained at −169 °C (lower trace); the sample was thawed and cyclopropanol was added, 2.8 mM. After 10 min at 25 °C the sample was frozen and the electron spin resonance spectrum obtained at −169 °C (upper trace). (Note: At 25 °C, inactivation occurs in the absence of NH_4^+.)

propanol to the enzyme in the absence of NH_4^+ leads to a change in the electron spin resonance spectrum as shown in Figure 5. The signal shape changes, especially at $g = 2.0045$. The addition of ammonium ion to the enzyme inactivated in this way results in only a small change in the electron spin resonance spectrum of the inactivated enzyme. Only a slight sharpening of the main peak at $g = 2.0045$ is observed upon ammonium ion addition to the enzyme. This is in contrast to the effect of ammonium ion on the spectrum of the enzyme prior to inactivation, as shown in Figures 3 and 4.

The experiment with [1-^{2}H]cyclopropanol indicates that the C-1 carbon–hydrogen bond is broken during the inactivation by cyclopropanol. In order to determine the fate of the C-1 hydrogen, we inactivated the enzyme with [1-^{3}H]cyclopropanol. A tritium isotope effect occurs during the inactivation. Therefore, for maximization of the reaction of enzyme with tritiated cyclopropanol, the inactivation was done with an excess of enzyme over cyclopropanol. The red form of the enzyme, sp act. 1.0 IU/mg, 0.91 μmol in 0.9 mL of 0.3 M sodium borate buffer, pH 9.0, containing 40 mM NH_4^+ ion, was inactivated with [1-^{3}H]cyclopropanol (0.076 μmol; 2.55×10^5 cpm/μmol). After 120 min, 65% of the enzyme activity was lost compared to a control in which cyclopropanol was omitted. Nonisotopic cyclopropanol (2 μmol) was then added, and the enzyme was placed on a G-25 column (1 × 20 cm) in the same buffer. The protein peak (void volume) contained 95% of the radioactivity (at constant specific activity throughout the peak) originally present in the cyclopropanol. The experiment was repeated several times, and the radioactive protein solutions eluted from the G-25 column were pooled. Aliquots of this pool were treated in several ways. To an aliquot (1865 cpm) was added 9 volumes of methanol. The denatured protein was removed and the supernatant fluid was found to contain 800 cpm (43%). The liquid was brought to dryness under reduced pressure, and all radioactivity was found in the residue, i.e., the radioactive material released from the enzyme is nonvolatile.

Urea, to final concentration of 6 M, was added to the remaining pool from the Sephadex column. An aliquot of this solution was again distilled under reduced pressure. No radioactive material was found in the distillate. Another aliquot (1.2 mL, containing 2160 cpm) was dialyzed against 3 changes of 6 M urea of 1.2 mL each. A total 700 cpm were released into three dialysates, i.e., a total of 32% of the radioactivity associated with the protein is dialyzable. A third aliquot containing 2500 cpm was applied to a 1 × 12 cm Sephadex G-25 column equilibrated in 6 M urea and eluted with the same solution. The protein, eluted at the void volume, contained 68% (1360 cpm vs. 22 cpm of background) of the tritium, and the small molecule fractions contained 32% of the tritium.

Table I: Deuterium Kinetic Isotope Effect on Methanol Dehydrogenase Reaction[a]

PMS concn (mM)	$V_{max}^{CH_3OH}/V_{max}^{CD_3OH}$
7.1	4.6
1.9	3.5
0.37	1.55
0.18	1.37

[a] Reaction rates were measured with an O_2 electrode under standard assay conditions, except that PMS was varied as indicated.

These results show that the reaction of [1-^{3}H]cyclopropanol does not lead to the formation of [^{3}H]H_2O; i.e., less than 5% of the tritium originally added is converted to [^{3}H]H_2O. The major fraction of the tritium, 60–70% originally present in cyclopropanol, is associated with the enzyme, is not removed by gel filtration or urea denaturations, and appears to be covalently attached. A fraction of the tritium (30–40%) appears to be present in a small molecule which is released from the enzyme upon denaturation.

Isotope Effects in the Oxidation of Methanol. The rate of oxidation of [$^2H_3$]CH_3OH was determined in the standard O_2-electrode assay, except that the concentration of PMS was varied. The results are summarized in Table I. At high PMS concentrations a deuterium isotope effect of 4.6 was seen. This isotope effect decreases to approximately 1 at low dye concentrations.

Oxidation State of the Enzyme. The possibility was considered that the enzyme as isolated is present in the reduced state. Addition of enzyme to PMS in the absence of substrate should therefore lead to O_2 consumption equivalent to the amount of enzyme present. This is not the case. Enzyme (200 nmol) in 0.25 M Tris buffer, pH 9.0, containing 16 mM NH_4^+ was placed into the compartment of the O_2 electrode. No O_2 consumption was detected after addition of 0.5 μmol of PMS. The consumption of 0.5 nmol of O_2 could have been detected.

Isotope-Exchange Experiments. The results obtained with cyclopropanol suggest that a chemical interaction between cyclopropanol and enzyme can occur in the absence of electron acceptor. To obtain additional evidence for a chemical reaction between the normal substrate and enzyme, we carried out a number of isotope-exchange experiments. If the equilibrium shown in eq 2 occurs, then hydrogens and electrons are

$$CH_3OH + E_{ox} \rightleftharpoons CH_2O + E_{red} \quad (2)$$

transferred from the substrate to the enzyme. The hydrogen transferred to the enzyme may or may not be exchangeable with solvent hydrogens. If it is exchangeable, then the enzyme should catalyze exchange of the hydrogens of methanol with solvent hydrogens.

To test this possibility, we incubated the red form of the enzyme, sp act. 1.0 IU/mg, 0.17 mM in 0.1 M sodium borate, pH 9.0, containing 40 mM NH_4Cl, at 25 °C with 1-[1-^{3}H]-butanol, 2.2 mM (1.9×10^5 cpm/μmol), in a total volume of 0.7 mL (V_{max} for 1-butanol is nearly equal to that of methanol). The solution of enzyme and butanol was kept at 25 °C for 90 min, and then 1 mmol of 1-butanol and 1 mmol of semicarbazide hydrochloride were added to the solution. The volatile substances were removed by bulb-to-bulb distillation at reduced pressure. The total distillate was examined by HPLC. This procedure separates water from alcohols. A maximum of 30 cpm was detected in the water peak. During the reaction period all of the 1-butanol could have been oxidized if an electron acceptor were present. Assuming that only

half the tritium is available for exchange and a ^3H isotope effect of 10 on the exchange, at least 2.4×10^3 cpm could be found in the water. Less than 1% of this amount was actually formed.

This result shows that alcohol dehydrogenase does not catalyze exchange of the substrate C-1 hydrogens with solvent protons to an appreciable extent. If hydrogen transfer from substrate to the enzyme occurs, then this hydrogen does not exchange with solvent protons. It should, however, be transferable to another substrate provided the product of alcohol oxidation is released from the enzyme. The above experiment was, therefore, repeated in the presence of 50 mM propionaldehyde. No [^3H]propanol was detected, i.e., less than 0.1% of the amount expected at equilibrium. Additional experiments were carried out in which ^3H incorporated into ethanol from [^3H]H_2O was determined in the absence and presence of acetaldehyde. Less than 1% of the tritium incorporation expected was observed.

Discussion

The unusual aspect of methoxatin-dependent methanol dehydrogenase is that addition of the substrate to the enzyme produces neither a change in the intensity of the electron spin resonance signal nor a change in the optical spectrum of the enzyme. This is in marked contrast to other oxidative enzymes, such as flavoproteins or NAD-dependent enzymes, where characteristic changes in the absorption spectra of the coenzyme are observed after substrate addition. This then raises the possibility that no reaction results when substrate is added to the enzyme in the absence of acceptor dyes. This would occur if the enzyme catalyzes the transfer of electrons from substrate to dye but does not itself serve as an electron acceptor (eq 3). Another possibility is that the enzyme, as isolated,

$$E \overset{S}{\rightleftharpoons} E^S \overset{dye_{ox}}{\rightleftharpoons} E^S_{dye_{ox}} \rightleftharpoons E^P_{dye_{red}} \overset{P,dye_{red}}{\rightleftharpoons} E \quad (3)$$

is in the reduced form and must be reoxidized by PMS before it can react with substrate. The data presented here allow both of these possibilities to be dismissed. Since no O_2 is taken up when PMS is added to the enzyme in the absence of substrate, the enzyme is not present in the reduced form. The mechanism shown in eq 3 is inconsistent with the deuterium isotope effect, i.e., the absence of isotope effect at low dye concentrations. This observation, however, is consistent with the mechanism shown in eq 4.

$$\begin{array}{l} E_{ox} \overset{S}{\rightleftharpoons} E^S_{ox} \rightleftharpoons E^P_{red} \overset{P}{\rightleftharpoons} E_{red} \overset{dye_{ox}\ dye_{red}}{\rightleftharpoons} E_{ox} \\ \qquad\qquad\qquad\qquad\qquad\qquad E^P_{ox} \overset{dye_{ox}\ dye_{red}}{\rightleftharpoons} E_{ox} \end{array} \quad (4)$$

We believe that the reaction with cyclopropanol, which occurs in the absence of an electron acceptor, also supports the formation of a reduced enzyme. In the inactivation of the enzyme by [1-^2H]cyclopropanol, a kinetic isotope effect occurs. This indicates that inactivation involves rupture of the carbon–hydrogen bond. It appears to us that this most probably occurs through an oxidative process.

The isotope-exchange experiments allow the reaction to be described in more detail. The lack of carbon exchange between alcohol and aldehyde indicates that the product aldehyde is not released from the reduced enzyme. Furthermore, the hydrogen transferred to the enzyme when the substrate is oxidized, in the absence of electron-acceptor dye, is not subject to exchange with solvent protons. The lower branch of the reaction sequence shown in eq 4, therefore, most appropriately describes the reaction catalyzed by methanol dehydrogenase.

We interpret the data we have obtained to indicate that only 14% of the isolated enzyme is catalytically active. This conclusion is based on the observation that the amount of cyclopropanol required to completely inactivate the enzyme is stoichiometrically equivalent to approximately 14% of the total enzyme present. The total enzyme concentration was estimated from either the amount of protein present or the total amount of coenzyme. If this interpretation is accepted, then other conclusions concerning the properties of this enzyme can be reached. The number of unpaired electrons detected by electron spin resonance is equivalent to 13% of the total enzyme. This suggests that only enzyme molecules that contain the semiquinone are catalytically active. This conclusion is supported by the observation that manipulations that lead to loss of the paramagnetic species also lead to concomitant loss of enzyme activity. Furthermore, the concentration of semiquinones is at least qualitatively related to the specific activity of the enzyme. If only 14% of the enzyme is catalytically active, then the major fraction of coenzyme present is irrelevant to the catalytic process. It can then be readily understood why relatively large changes in the spectrum of the holoenzyme are not associated with changes in specific activity and why spectral changes that might occur upon addition of substrate might not be observed.

The data available so far are not sufficient to completely define the mechanism of inactivation by cyclopropanol. As already stated, we believe cyclopropanol is oxidized by the enzyme. In this respect the reaction of cyclopropanol is analogous to oxidation of the normal substrate. However, unlike the normal substrate, the product derived from the oxidation of cyclopropanol is unstable and decomposes. This tends to drive the oxidation to completion. We do not know the nature of the oxidation product(s) derived from cyclopropanol, except that it is not H_2O.

If interaction of substrate and coenzyme in the absence of electron-acceptor dye results in oxidation of the substrate, then the enzyme–coenzyme complex must be reduced. What is the electron acceptor? Possibly the semiquinone form of the cofactor undergoes a two-electron reduction.[3] Such a process would preserve its paramagnetism.

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[3] Professor R. W. Johnson, Department of Chemistry, Harvard University, has obtained preliminary evidence that indicates that a quinone closely related to methoxatin can undergo a three-electron reduction.

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Mechanistic Studies on the Pyridoxal Phosphate Enzyme 1-Aminocyclopropane-1-carboxylate Deaminase from *Pseudomonas* sp.†

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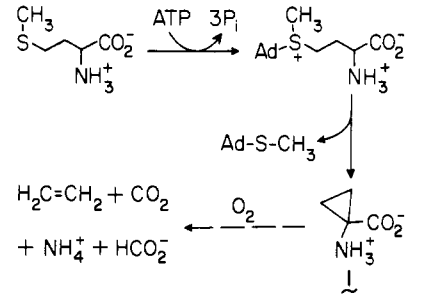
ABSTRACT: The enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACPC deaminase) from a pseudomonad is a pyridoxal phosphate (PLP) linked catalyst which fragments the cyclopropane substrate to α-ketobutyrate and ammonia [Honma, M., & Shimomura, T. (1978) *Agric. Biol. Chem. 42*, 1825]. Enzymatic incubations in D_2O yield α-ketobutyrate with one deuterium at the C-4 methyl group and one deuterium at one of the C-3 prochiral methylene hydrogens. Stereochemical analysis of the location of the C-3 deuteron was accomplished by in situ enzymatic reduction to (2*S*)-2-hydroxybutyrate with L-lactate dehydrogenase and conversion to the phenacyl ester. The C-3 hydrogens of the (2*S*)-2-hydroxybutyryl moiety are fully resolved in a 250-MHz NMR spectrum. Absolute assignment of 3*S* and 3*R* loci was obtained with phenacyl (2*S*,3*S*)-2-hydroxy[3-^{2}H]butyrate generated enzymatically by D-serine dehydratase action on D-threonine. ACPC deaminase shows a stereoselective outcome with a 3*R*:3*S* deuterated product ratio of 72:28. 2-Vinyl-ACPC is also a fragmentation substrate with exclusive regiospecific cleavage to yield the straight-chain keto acid product 2-keto-5-hexenoate. The D isomer of vinylglycine is processed to α-ketobutyrate and ammonia at 8% the V_{max} of ACPC, while L-vinylglycine is not a substrate. It is likely that ACPC and D-vinylglycine yield a common intermediate—the vinylglycine–PLP–*p*-quinoid adduct—which is then protonated sequentially at C-4 and then C-3 to account for the observed deuterium incorporation. The D isomers of β-substituted alanines (fluoroalanine, chloroalanine, and *O*-acetyl-D-serine) partition between catalytic elimination and enzyme inactivation. Each shows a different partition ratio, arguing against the common aminoacrylyl-PLP as the inactivating species.

The cyclopropanoid amino acid 1-aminocyclopropane-1-carboxylic acid (ACPC)[1] (**1**) is a natural product isolated from several plant tissues (Burroughs, 1957; Vahatalo & Virtanen, 1957) including pears and apples. This cyclic amino acid is a key intermediate in the biosynthesis of ethylene, a fruit-ripening hormone in plants (Adams & Yang, 1979; Lurssen et al., 1979; Konze & Kende, 1979), and consequently there has been interest in the biotransformations to and from ACPC. A likely route for ethylene biosynthesis from methionine (Scheme I) commences with S-adenosylation to give *S*-

Scheme I



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[1] Abbreviations used: ACPC deaminase, 1-aminocyclopropane-1-carboxylate deaminase; PLP, pyridoxal 5′-phosphate; SAM, *S*-adenosylmethionine; Me_4Si, tetramethylsilane; TSP, 3-trimethylsilyl-[2,2,3,3-$^2H_4$]propionate; LDA, lithium diisopropylamide; THF, tetrahydrofuran; ORD, optical rotatory dispersion.

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