Mechanism of Action of Methanol Oxidase, Reconstitution of Methanol Oxidase with 5-Deazaflavin, and Inactivation of Methanol Oxidase by Cyclopropanol†

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ABSTRACT: Methanol oxidase isolated from *Hansenula polymorpha* contains two distinct flavin cofactors in approximately equal amounts. One has been identified as authentic FAD and the other as a modified form of FAD differing only in the ribityl portion of the ribityldiphosphoadenosine side chain. The significance of this finding is as yet unknown. Previous studies have shown that cyclopropanol irreversibly inactivates methanol oxidase [Mincey, T., Tayrien, G., Mildvan, A. S., & Abeles, R. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7099–7101]. We have now established that inactivation is accompanied by covalent modification of the flavin cofactor. The stoichiometry of this reaction is 1 mol of cyclopropanol/mol of active flavin. The structure of the covalent adduct was determined by NMR, IR, and UV spectral studies to be an N_5,C_{4a}-cyclic 4a,5-dihydroflavin. Reduction of the covalent adduct with $NaBH_4$ at pH 9.0 before removal from the enzyme converted it to the 1-(ribityldiphosphoadenosine)-substituted 4-(3-hydroxypropyl)-2,3-dioxoquinoxaline. Cyclopropyl ring cleavage accompanies inactivation, and covalent bond formation occurs between a methylene carbon of cyclopropanol and N_5 of flavin. Methanol oxidase was also reconstituted with 5-deazaflavin adenine dinucleotide (dFAD). Reconstituted enzyme did not catalyze the oxidation of alcohols to the corresponding aldehydes, nor did reduced reconstituted enzyme catalyze the reverse reaction. Incubation of reconstituted enzyme with cyclopropanol resulted in an absorbance decrease at 399 nm, but no irreversible covalent modification of the deazaflavin cofactor. A reversible addition complex between cyclopropanol and dFAD is formed. The structure of that complex was not definitively established, but it is likely that it is formed through the addition of cyclopropoxide to C_5 of dFAD. The failure of dFAD-reconstituted methanol oxidase to catalyze the oxidation of substrate, as well as the lack of reaction with cyclopropanol, supports a radical mechanism for alcohol oxidation and cyclopropanol inactivation. Methanol oxidase catalyzes the oxidation of cyclopropylcarbinol to the corresponding aldehyde. No ring-opened products were detected. The failure to form ring-opened products has been used as an argument against radical processes [MacInnes, I., Nonhebel, D. C., Orsculik, S. T., & Suckling, C. J. (1982) *J. Chem. Soc., Chem. Commun.*, 121–122]. We present arguments against this interpretation.

Flavoproteins are known to carry out two types of alcohol oxidations: (1) those in which the hydrogen removed is relatively acidic due to its position α to a carbonyl group and (2) those in which the hydrogen removed is not thus activated. Lactate oxidase falls within the first category. Chemical model studies show that reactions of this type are initiated by abstraction of the hydrogen as a proton and formation of a carbanion (Bruice, 1975). Studies with lactate oxidase are consistent with a carbanion mechanism (Walsh et al., 1973). In contrast, chemical model studies of reactions which fall within the second category show that oxidation proceeds through a radical mechanism (Williams et al., 1975). Analogous enzyme-catalyzed reactions have been less extensively studied. Evidence for radical involvement has been obtained for a closely related reaction, the oxidation of amines catalyzed by mitochondrial monoamine oxidase (Silverman et al., 1980; Silverman & Hoffman, 1981).

Methanol oxidase is an example of a flavoprotein in which an unactivated hydrogen is removed during the course of oxidation. Methanol oxidase is purified from the yeast *Hansenula polymorpha* and catalyzes the oxidation of short-chain primary alcohols to the corresponding aldehydes with the concomitant reduction of molecular oxygen to hydrogen peroxide (Kato et al., 1976). In the cell, methanol oxidase and catalase are localized in peroxisomes where hydrogen peroxide generated upon methanol oxidation is immediately metabolized to O_2 and H_2O by catalase (Anthony, 1982). Methanol oxidase has been purified to homogeneity. The enzyme is an octamer of M_r 667 000 containing 7 mol of flavin/mol of enzyme (Kato et al., 1976). Only two of the seven flavins present are in the oxidized state and participate in oxidation–reduction reactions. The other five flavins are present as the stable red semiquinone and appear inert in the catalytic process (Mincey et al., 1980). Cyclopropanol irreversibly inactivates methanol oxidase (Mincey et al., 1980). Initial inactivation studies using 1-[^{3}H]cyclopropanol resulted in a radioactive flavin derivative which could be separated from the enzyme. The structure of the covalent adduct formed between flavin and cyclopropanol has now been established.

We report, herein, studies on the mechanism of action of methanol oxidase and the mechanism of inactivation of methanol oxidase by cyclopropanol. We believe that both alcohol oxidation and cyclopropanol inactivation have common mechanistic features and that elucidation of the latter process will provide information pertinent to the catalytic mechanism.

Experimental Procedures

Chemicals. D- and L-lyxoflavins were obtained from Dr. Donald McCormick. Cyclopropanol, 1-[^{2}H]cyclopropanol, and 1-[^{3}H]cyclopropanol were synthesized and purified as de-

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scribed previously (Parkes & Abeles, 1984). NaB^3H_4 was obtained from New England Nuclear, NaB^2H_4 from Merck, ^2H_2O from Bio-Rad, and ACS for scintillation counting from Amersham. All other chemicals were reagent grade and obtained from Aldrich Chemical Co. or Sigma Chemical Co. Urea was recrystallized 2× from 95% ethanol.

Enzyme Purification. Methanol oxidase was obtained from two sources. Unless noted, all experiments were performed with methanol oxidase isolated from the yeast *Hansenula polymorpha* (ATCC 26012) and purified by modification of published procedures (Kato et al., 1976). Purified enzyme preparations were >95% homogeneous by sodium dodecyl sulfate (SDS) gel electrophoresis and had specific activities ranging from 11 to 17 IU/mg of protein. Enzyme from this source is an octamer of M_r 640 000 containing 5 mol of semiquinone and 2 mol of oxidized flavin per mol of enzyme. Cofactor composition is approximately 51% FAD I and 49% FAD.

Final NMR decoupling experiments, as indicated, were performed on commercial methanol oxidase EC 1.1.3.13 (Calbiochem). This enzyme was isolated and purified from the yeast *Pichia pastoris* according to published procedures (Kato et al., 1976). It is an octamer of M_r 635 000 containing 5 mol of semiquinone and 3 mol of oxidized flavin per mol of enzyme. Cofactor composition is 87% FAD I and 13% FAD. It has a specific activity of 18 IU/mg of protein.

Measurement of Enzyme Activity. Methanol oxidase activity was assayed by CH_3OH-dependent O_2 consumption with an oxygen electrode. Standard assays were carried out at 37 °C. Assay mixtures contained 6 mM CH_3OH, 0.3 μM catalase, 0.05 M potassium phosphate buffer (pH 7.5), and methanol oxidase ranging from 0.02 to 0.2 μM. One unit of methanol oxidase is defined as the amount of enzyme able to consume 1 μmol of O_2/min.

Determination of Active Flavin. When enzyme concentration is given as moles of active flavin per liter, "active" flavin refers to the two oxidized flavins per octamer that are reduced by the addition of substrate. The concentration of active flavin was determined by anaerobic titration of methanol oxidase with CH_3OH in 50 mM potassium phosphate buffer (pH 7.5) at 25 °C. The total decrease in 450-nm absorbance observed divided by 10.2×10^3 M^{-1} cm^{-1} (difference between the extinction coefficient of FAD and that of $FADH_2$ at 450 nm) yielded the concentration of active flavin in moles per liter.

Inactivation of Methanol Oxidase with Cyclopropanol. Methanol oxidase (17 μM) in 0.05 M potassium phosphate buffer (pH 7.5) was incubated with cyclopropanol (100 μM) for 60 min at 25 °C. The UV–visible absorption spectrum was monitored to verify that the decrease in 450-nm absorbance was complete.

Isolation of Adduct I. Enzyme inactivated with cyclopropanol as described above was diluted 1:1 with a 6 M urea/3.5 M KBr solution and incubated at 37 °C for 20 min. Partially denatured enzyme was applied to a Sephadex G-25 column (1 × 22 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 7.5), and eluted at a flow rate of 0.5 mL/min. Protein-containing fractions (as determined by absorbance at 280 nm) were pooled, and protein was precipitated by incubation with 3 volumes of CH_3OH for 18 h at 4 °C. Precipitated protein was removed by centrifugation at 40000*g* and 4 °C for 30 min. Modified flavin containing supernatant fluid was concentrated to 1.0 mL by passing a slow stream of N_2 through the sample. Desalting was accomplished by applying sample to a C_{18} Sep-pak (Waters Associates) prewashed with distilled water (to which modified flavin tightly binds), washing with 2.0 mL of distilled water, and finally eluting modified flavin with 2.5 mL of 60:40 methanol/distilled water mixture. Eluate was concentrated as above to 0.5 mL and chromatographed by reverse-phase high-performance liquid chromatography (HPLC) (system B). Modified flavin was visualized by its UV absorbance at 254 nm. All isolation procedures were carried out in the absence of any direct lighting.

Isolation of Adduct III. Methanol oxidase was inactivated with cyclopropanol as described. When inactivation was >96% complete, the pH of the inactivation mixture was adjusted to 9.0 with KOH, and 2.5 mg of solid $NaBH_4$ was added. After 10 min, an additional 2.5 mg of solid $NaBH_4$ was added. The pH was maintained at 9.0 throughout the reduction which was carried out in the dark. After 30 min, 0.01 mL of acetone was added to react with any remaining $NaBH_4$. The reduced mixture was diluted 1:1 with a 6 M urea/3.5 M KBr solution and then treated according to the procedure described for adduct I.

Experimental Determination of Extinction Coefficients. Extinction coefficients for all covalently modified flavins were determined as follows. Methanol oxidase was inactivated with either ^3H- or ^{14}C-labeled cyclopropanol of known specific activity. Each radiolabeled adduct species was purified to a single peak on HPLC as described. The radioactivity contained in each sample was determined by scintillation counting. Since the specific activity of the cyclopropanol is known, the concentration of flavin adduct in moles per liter can be calculated. The absorbance at λ_{max} for each sample was also measured, and absorbance at λ_{max} divided by concentration of adduct in moles per liter yields the extinction coefficient at λ_{max} in M^{-1} cm^{-1}.

pK_a Determination. pK_a's were determined by spectral titration. Modified flavin species were diluted to 50 μM with 1 M KCl. The pH of the solution was then titrated by addition of microliter increments of 1 N HCl or 1 N NaOH. Optical spectra were recorded after each addition. Isobestic points were observed spectrally. pK_a values were determined by plotting absorbance changes at a fixed wavelength vs. pH.

Enzymatic Synthesis of 5-Deazaflavin. 5-Deazariboflavin was a gift from Dr. Louis B. Hersh. 5-Deazariboflavin was phosphorylated and adenylated enzymatically with FAD synthetase complex purified from *Brevibacterium ammoniagenes* (ATCC 6872) according to published procedures (Spencer et al., 1976) with the following modifications. First, cells were disrupted by homogenization for a total of 9 min (6 × 1.5-min cycles) in a Biospec Products Bead-Beater 3/4 filled with 0.1–0.15-mm glass beads. Second, following G-100 column chromatography, adenylating activity was further purified on a Pharmacia FPLC column (system G). Activity eluted at 0.35 M potassium phosphate buffer (pH 7.5). Third, 5-deazaflavin which had been purified by ion-exchange chromatography as described by Spencer et al. (1976) was chromatographed by reverse-phase HPLC (system B) to remove small amounts of contaminating 5-deazaFMN. 5-Deazaflavin concentrations were determined by using an ϵ_{399} of 11.5×10^3 M^{-1} cm^{-1} for dFAD.

Reconstitution of Enzyme with 5-Deazaflavin. A total of 0.095 mL of methanol oxidase (73 μM) was diluted to 0.29 mL with 0.05 M potassium phosphate buffer (pH 7.5) containing 6 M urea and 3.5 M KBr. This mixture was incubated at 25 °C in the dark. Its UV–visible absorption spectrum was monitored during this time, and when all flavin cofactor had been removed from the enzyme (evidenced by the oxidation of all flavin semiquinone), the mixture was applied to a

Sephadex G-25 column (1 × 9 cm) prepared as follows. The column was equilibrated with 0.03 M potassium phosphate buffer (pH 7.8) containing 6 M urea. To the column was added 0.25 mL of 4 mM dFAD, and the dFAD was allowed to migrate exactly 3 cm from the top of the column. The enzyme was then applied, the column flow rate was regulated at 0.45 mL/min and 45-s fractions were collected. Protein-containing fractions (determined by absorbance at 280 nm) were pooled and dialyzed for 8 h against 3 × 1 L of 0.03 M potassium phosphate buffer (pH 7.5) followed by 8 h against 3 × 1 L of 0.02 M sodium pyrophosphate/0.01 M KCl buffer (pH 8.6). Upon completion of dialysis all samples were recovered, diluted to equal volumes with 0.02 M sodium pyrophosphate/0.01 M KCl buffer (pH 8.6), and clarified by centrifugation for 25 min at 20000*g* and 4 °C. Supernatant fluid from each sample was removed and stored in the dark at 4 °C.

Reduced 5-Deazaflavin Reoxidation in the Presence of Aldehydes. For enzymatic studies, 1.0 mL of dFAD-reconstituted methanol oxidase (1.2 μM) was transferred to an anaerobic cuvette. For nonenzymatic studies, 1.0 mL of dFAD (9.6 μM) was transferred to an anaerobic cuvette. The cuvette was sealed and the solution flushed with argon for 30 min. The UV spectrum was recorded. Sample was then titrated anaerobically with sodium dithionite (sequential 2-μL additions of a 5.1 g/L stock solution of NaS_2O_4) until further addition of NaS_2O_4 caused no change in 399-nm absorbance. The UV spectrum was recorded after each addition. After complete reduction of dFAD, appropriate amounts of aldehyde dissolved in 0.01 mL were added anaerobically to the system. For spectral studies, at t_0 after aldehyde addition repetitive scanning was begun. Scans between 800 and 200 nm were recorded at 8-min intervals. For kinetic studies, the change in absorbance at 410 nm was recorded over time. Semilog plots of percent change in absorbance at 410 nm vs. time for each concentration of aldehyde yielded $t_{1/2}$ for reoxidation. k_{obsd} at each concentration was calculated by using the equation $k_{obsd} = 0.693/t_{1/2}$. For each aldehyde, second-order rate constants were determined from the slope of a plot of k_{obsd} vs. aldehyde concentration.

UV–Visible Spectroscopy. Optical spectra were recorded on a Perkin-Elmer 559 spectrophotometer thermostated at 25 °C unless otherwise specified.

Proton Magnetic Resonance Spectroscopy. Proton NMR spectra were recorded on a Bruker WH 90-MHz FT NMR at a sweep width of 1200 hz. Spectra of FMN I and covalent adducts formed between flavin cofactor and cyclopropanol were recorded on either a 270-MHz FT NMR or a 500-MHz FT NMR at sweep widths ranging from 1000 to 4000 Hz. For decoupling experiments a 270-MHz FT NMR was used. The power of irradiating pulse was chosen so as to diminish the proton signal by approximately 80%. Data were recorded as difference spectra (spectra recorded while irradiating at resonance frequency far from the region of interest minus spectra recorded while irradiating at resonance frequency of the nucleus to be decoupled). Chemical shifts were measured in parts per million with solvent signal as the internal standard (HDO = 4.8 ppm). All samples were desalted on reverse-phase HPLC (system C), exchanged 3× into D_2O, redissolved in 0.2 mL of D_2O, and transferred to 0.2 mL volume microcells.

Infrared Spectroscopy. Infrared measurements were made by using a Nicolet 7199 Fourier transform (FT) infrared spectrometer purged with N_2. A sample cell consisting of CaF_2 windows separated by Teflon spacers of 0.1-mm thickness was used. Deuterium oxide which does not absorb in the region of interest (1550–2000 cm^{-1}) was employed as solvent. The double-beam IR spectrum of adduct III′ was obtained by dividing the single-beam spectrum of adduct III′ in D_2O by the single-beam spectrum of a D_2O reference sample. Each single-beam spectrum was an average of 500 interferograms. The sample consisted of adduct III′ which had been HPLC purified and desalted as described above, lyophilized, and exchanged 4× into D_2O (99.8 atom % D). After the final lyophilization, the sample was diluted to 40 μL with D_2O and stored at 4 °C.

Chromatographic Systems. The following were the conditions for each system: system A, C_{18} reverse-phase HPLC column (0.39 i.d. × 30 cm, Waters Associates), isocratic elution at 2.0 mL/min, solvent distilled water; system B, C_{18} reverse-phase HPLC column (0.39 i.d. × 30 cm, Waters Associates), gradient elution at 1.6 mL/minute, solvent A 10 mM ammonium acetate buffer (pH 5.8), solvent B CH_3OH, 5-min isocratic elution with 20% solvent B followed by 72-min linear gradient to 100% solvent B; system C, C_{18} reverse-phase HPLC column (0.39 i.d. × 30 cm, Waters Associates), gradient elution at 1.0 mL/min, solvent A distilled water, solvent B CH_3OH, 15-min isocratic elution with 0% solvent B followed by 15-min linear gradient to 100% solvent B; system D, Bio-Rad Organic Acids column HPX 87 (0.78 × 30 cm), isocratic elution at 0.6 mL/min, solvent 5 mM H_2SO_4; system E, cellulose TLC sheets, solvent system 1-butanol/acetone/acetic acid/5% NH_4OH/water (35 + 25 + 15 + 15 + 10); system F, descending paper chromatography (Whatman No. 1), solvent cyclohexane; system G, Mono Q fast protein liquid chromatography column (HR 5/5, Pharmacia Fine Chemicals) gradient elution at 1.0 mL/min, solvent A 0.05 M potassium phosphate buffer (pH 7.5), solvent B 1.0 M potassium phosphate buffer (pH 7.5), 30-min linear gradient from 0% solvent B to 100% solvent B; system H, C_{18} reverse-phase HPLC column (0.39 i.d. × 30 cm, Waters Associates), isocratic elution at 1.5 mL/min, solvent acetonitrile/distilled water (1:1).

Phosphodiesterase I Cleavage. A total of 0.1–5.0 μmol of FAD or covalently modified FAD was diluted to 0.2 mL with distilled water. After pH was adjusted to 8.4 with 0.1 M NH_4OH, 0.1 mg of phosphodiesterase I (*Crotolus adamanteous* venom, 0.11 unit/mg) in 0.05 mL of distilled water was added, and the cleavage reaction was allowed to proceed for 20 min at 37 °C in the dark. The reaction was stopped at 20 min by adjusting pH to 6.0 with 0.1 M acetic acid. To precipitate protein, 3 volumes of CH_3OH was added, and the mixture was allowed to sit 10 min at 4 °C. The mixture was centrifuged at 20000*g* and room temperature for 10 min. Reaction products were purified by reverse-phase HPLC (system B).

Alkaline Phosphatase Cleavage. A total of 0.1–5.0 μmol of FAD or covalently modified FAD was treated as described above for phosphodiesterase I cleavage with the following modification. Before the reaction was stopped by acidification, 5 μL of alkaline phosphatase (300 units/mL) was added to the mixture and allowed to react at 37 °C for 30 min.

Results

Cofactor Composition of Methanol Oxidase. The flavin cofactor from *H. polymorpha* methanol oxidase was resolved from the protein and examined by HPLC (system B). Two distinct peaks were detected with retention times of 13.8 and 16.2 min, respectively (Figure 1). The two peaks will be designated FAD I (13.8 min) and FAD II (16.2 min). Flavin cofactor similarly resolved from D-amino acid oxidase elutes as a single peak on HPLC with a retention time of 16.2 min.

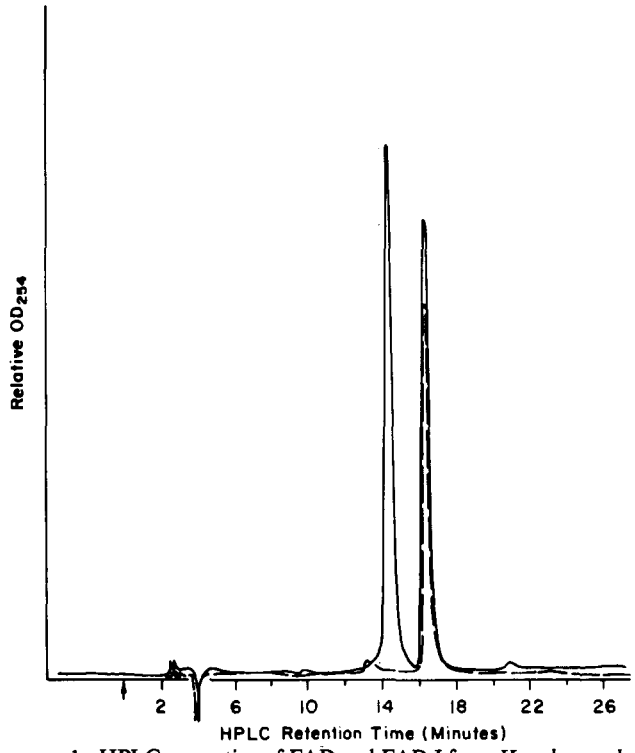


FIGURE 1: HPLC separation of FAD and FAD I from *H. polymorpha* methanol oxidase. Flavin cofactor was isolated from 5.0 nmol of methanol oxidase as described under Experimental Procedures and applied to a Waters C_{18} reverse-phase HPLC column (0.39 i.d. × 30 cm) and chromatographed by using system B (—). A total of 5.0 nmol of commercial FAD was chromatographed under identical conditions (- - -).

The two peaks observed upon chromatography of flavin from methanol oxidase are, therefore, not due to an artifact of isolation, and it is concluded that methanol oxidase from *H. polymorpha* contains two distinct flavin species.

The properties of the two flavins were examined further. The UV spectrum of FAD I is identical with that of authentic FAD, except that the extinction coefficient at 370 nm is slightly larger relative to that at 445 nm. The spectrum of FAD II is identical with that of FAD. Treatment of FAD I and FAD II with phosphodiesterase I resulted in altered HPLC retention times. AMF was identified by thin-layer chromatography (TLC) (system E) and HPLC (system B) as a hydrolysis product from the cleavage of both species. The product obtained from FAD II after cleavage of AMP comigrates with FMN (retention time 19.9 min), while the product from FAD I migrates with a retention time of 18.6 min. These results show that FAD II is FAD. Removal of the terminal phosphate moiety with alkaline phosphatase led to further alteration in retention times, but the products obtained from FAD I and FAD II were not identical. However, treatment of both cofactors with $NaIO_4$ generated products of identical HPLC retention time (35.0 min) which comigrate with the product obtained when FMN was treated with $NaIO_4$. These results localize the difference between FAD I and FAD II (FAD) to the N_{10}-ribityl side chain. NMR spectra of both species support this conclusion. It is apparent from these spectra that the isoalloxazine ring structure is identical in FAD and FAD I. The ribityl regions of both chromophores contain seven protons, but their chemical shifts are distinctly different. The total number of protons, the corresponding chemical shift values, and the enzymatic cleavage properties of the N_{10}-ribityl side chain of FAD and FAD I suggest that the two species are optical isomers. FAD I was cleaved to the riboflavin level and compared with D- and L-lyxoflavin by HPLC (system B) and is distinct from both.

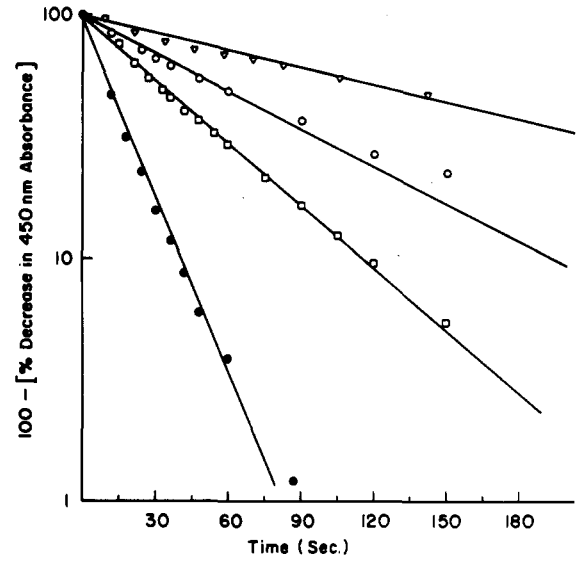


FIGURE 2: Pseudo-first-order decrease in 450-nm absorbance observed upon incubation of methanol oxidase with cyclopropanol. Reaction mixtures contained methanol oxidase (33 μM) in 1.0 mL of 0.05 M potassium phosphate buffer, pH 7.5. All incubations were carried out at 25 °C. Initial absorbance at 450 nm was recorded. Cyclopropanol was then added to final concentrations of 0.1 (▽), 0.2 (○), 0.4 (□), and 1.0 (●) mM, and absorbance at 450 nm was monitored as a function of time.

In contrast to *H. polymorpha* methanol oxidase where FAD and FAD I exist in roughly equivalent proportions (49% and 51%, respectively), *P. pastoris* methanol oxidase contains 13% FAD and 87% FAD I.

Inactivation of Methanol Oxidase by Cyclopropanol. Methanol oxidase is rapidly inactivated by cyclopropanol in a time-dependent manner. At cyclopropanol concentrations ≥0.2 mM inactivation is a pseudo-first-order process. No oxygen uptake is observed throughout the course of inactivation. In addition, incubation of cyclopropanol with enzyme results in a time-dependent, pseudo-first-order decrease in absorbance at 450 nm (Figure 2). The extent of this decrease is identical with the decrease in absorbance at 450 nm observed when enzyme-bound flavin is reduced by substrate under anaerobic conditions (Figure 3). The kinetics of cyclopropanol inactivation and bleaching of 450-nm absorbance were found to be the same. Inactivation was, therefore, further analyzed by measuring the rate at which 450-nm absorbance decreases following the addition of cyclopropanol. Saturation kinetics are observed. K_{cat} at saturation, measured at 5 °C, is 0.40 s^{-1}, and the cyclopropanol concentration at which one observes half-maximal rate is 8.7 mM.

The presence of substrate protects against inactivation, suggesting that inactivation is active site directed. No deuterium isotope effect is observed when enzyme is inactivated with 1-[^{2}H]cyclopropanol. At cyclopropanol concentrations less than 0.2 mM the kinetics of inactivation appear to become biphasic but were not investigated further. Inactivation is irreversible as evidenced by the failure of inactivated enzyme to regain activity after either extensive dialysis or passage through a Sephadex G-25 column.

Isolation of a Modified Flavin from Methanol Oxidase Inactivated with Cyclopropanol. Methanol oxidase (17 μM) was allowed to react with 1-[^{3}H]cyclopropanol (100 μM) for 60 min. At this point the enzyme had lost more than 96% of its catalytic activity. The inactive enzyme was passed through a Sephadex G-25 column (22 × 1 cm) to separate protein from excess inactivator. The protein peak was found to contain

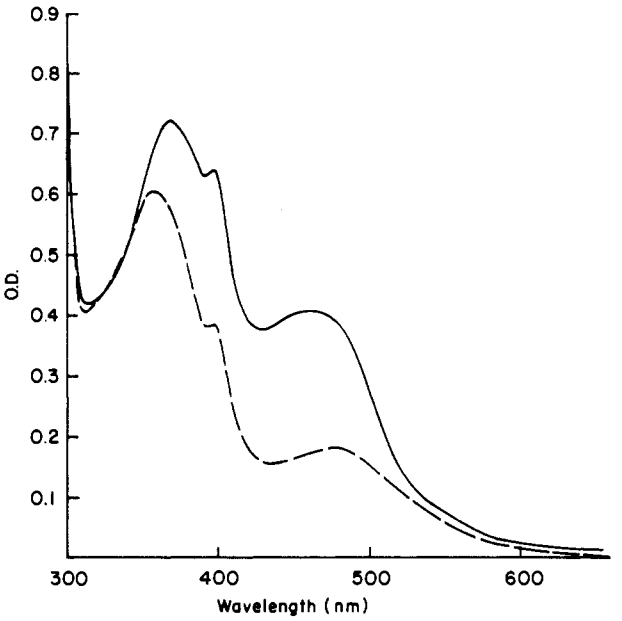


FIGURE 3: Absorption spectra of methanol oxidase alone and after the addition of either methanol or cyclopropanol. Native methanol oxidase (10.5 μM) (—). Methanol oxidase (10.5 μM) incubated with 6 mM methanol for 10 min under anaerobic conditions (---); methanol oxidase (10.5 μM) incubated with 200 μM cyclopropanol for 30 min (---); all spectra recorded at 25 °C in 0.05 M potassium phosphate buffer, pH 7.5.

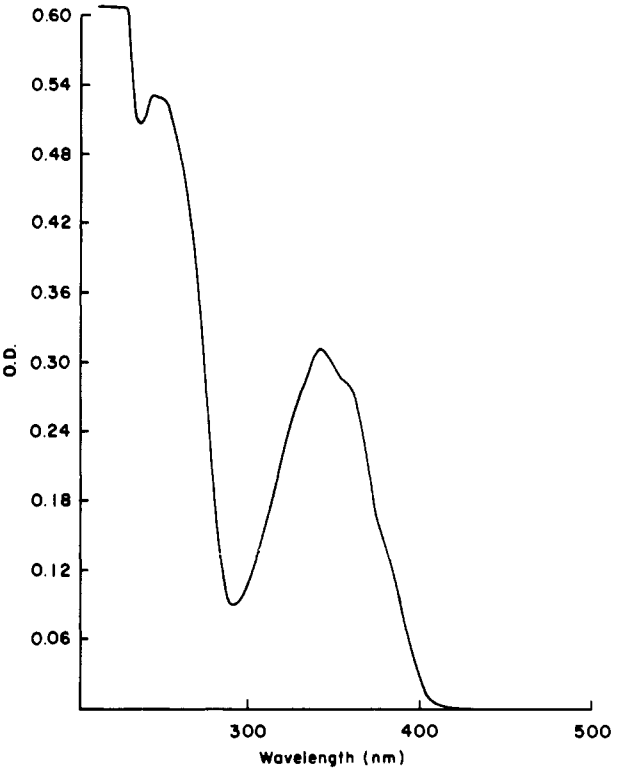


FIGURE 4: Absorption spectrum of adduct I. Adduct I (38.5 μM), isolated and purified as described under Experimental Procedures, in 0.05 M potassium phosphate buffer pH 7.5.

radioactive material corresponding to 2.3 mol of inactivator/mol of enzyme. Since only two of the seven flavins appear involved in catalysis, close to 1 mol of cyclopropanol has reacted per mole of active flavin. A similar experiment was carried out with [1-^{14}C]cyclopropanol. Radioactivity corresponding to 2.0 mol of cyclopropanol/mol of enzyme was found to comigrate with protein on Sephadex G-25.

These results, plus the observation that an irreversible decrease in 450-nm absorbance accompanies cyclopropanol inactivation, suggested the formation of a covalent adduct between flavin and cyclopropanol. Experiments were carried out to isolate such an adduct. Methanol oxidase (17 μM) was inactivated with 1-[^{3}H]cyclopropanol (100 μM) and then exposed to 3 M urea and 1.8 M KBr as described under Experimental Procedures. The partially denatured protein was applied to a Sephadex G-25 column, and two peaks of radioactivity were observed. The first peak was associated with the protein fraction. It was found to contain 1.3 mol of cyclopropanol/mol of active flavin. Spectral examination of this fraction indicated that the cyclopropanol–flavin adduct remained protein bound. The second peak was associated with the small molecule fraction. It was found to contain unmodified flavin derived from methanol oxidase and excess 1-[^{3}H]cyclopropanol. It was therefore concluded that urea–KBr treatment resolves the major portion of unmodified flavin from the protein, but not the cyclopropanol–flavin adduct.

In order to isolate the cyclopropanol–flavin adduct, the protein was precipitated by addition of 3 volumes of methanol. All of the radioactivity was recovered in the supernatant fluid. After desalting as described under Experimental Procedures, supernatant fluid was examined by reverse-phase HPLC (system B). Greater than 66% of radioactive label elutes in a broad peak with a retention time of 18.1 min, while residual unmodified FAD I and FAD elute at 13.8 and 16.2 min, respectively. Similar results were obtained in an experiment in which [1-^{14}C]cyclopropanol was used. These results establish that the flavin cyclopropanol adduct contains the three carbons and the C-1 hydrogen of cyclopropanol.

Characterization of Covalently Modified Flavin. The radioactively labeled material eluting from reverse-phase HPLC column at 18.1 min (referred to subsequently as adduct I) has a UV absorbance maximum at 342 nm and no absorbance above 400 nm (Figure 4), consistent with a two-electron-reduced flavin species. Adduct I is stable to oxidation by O_2. Assuming that 1 mol of cyclopropanol is covalently bound per mole of active flavin, an experimental extinction coefficient at 342 nm of 8.1×10^3 M^{-1} cm^{-1} was determined as described under Experimental Procedures. Adduct I has a pK_a at 10.5 (determined by spectral titration as described under Experimental Procedures), which can be assigned to protonation at N_3 (Walker et al., 1967), and suggests that the flavin C ring is intact. The absence of a pK_a between 4 and 8 argues against a 1,5-dihydroflavin structure with a double bond between C_{4a} and C_{10a} (Ghisla et al., 1974). In 6 N HCl the UV maximum remains at 342 nm, but the peak is broadened considerably, a characteristic of N_5,C_{4a}-disubstituted flavins. Adduct I is acid labile and undergoes a slow irreversible change upon acidification to pH 2.0. This change is visualized spectrally by a 19-nm shift in UV maxima to 223 nm. This species has not been characterized. Adduct I is also extremely labile to base. Acid lability and base lability are characteristic of N_5,C_{4a}-bridged flavins (Ghisla et al., 1974).

The N_{10}-side chain of FAD is retained in adduct I as evidenced by the release of adenosine monophosphate upon treatment with phosphodiesterase I and the release of inorganic phosphate upon subsequent reaction with alkaline phosphatase. No loss of radioactivity occurs upon cleavage by phosphodiesterase, alkaline phosphatase, or sodium periodate, indicating that the three carbons originating from cyclopropanol are covalently bound to the isoalloxazine ring moiety.

Rechromatography of adduct I on HPLC indicates that it consists of two labeled species, a major and a minor compo-

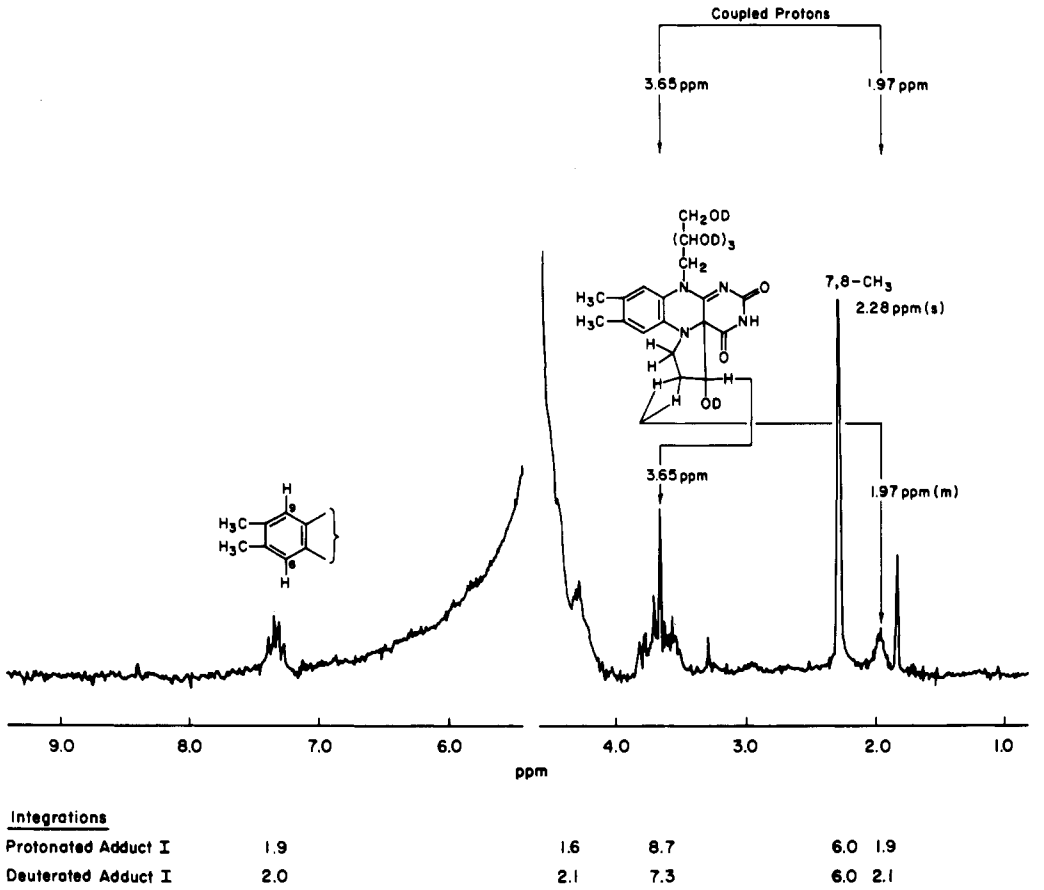


FIGURE 5: 270-MHz FT NMR spectrum of adduct I. NMR samples were prepared as described under Experimental Procedures. Chemical shift data, integrations, and decoupling studies are discussed in the text. Integration values are the average of two separate NMR experiments.

nent. If either species is reinjected immediately onto HPLC, it remains a single peak, but reinjection of either species after storage at 4 °C for >6 h results in the two original peaks. Possibly, the two species are in slow equilibrium with one another.

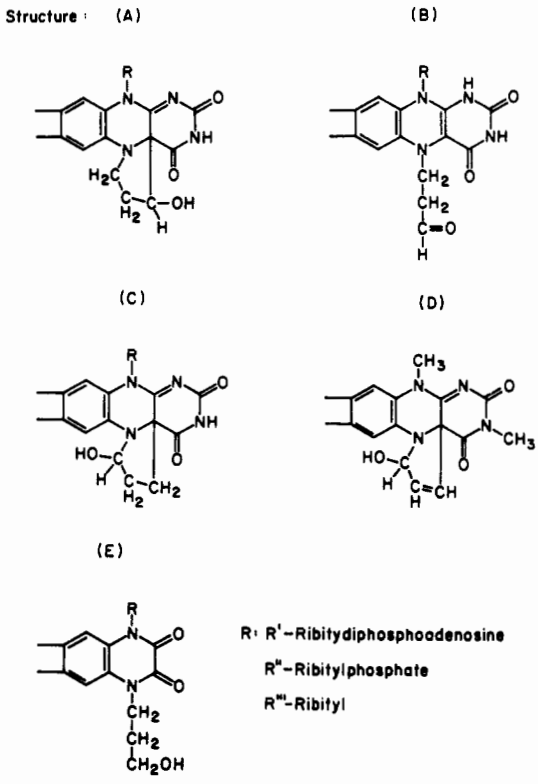
All evidence presented so far is consistent with the major adduct species being an N_5,C_{4a}-cyclic structure but does not prove such a structure. Further evidence supporting an N_5,C_{4a}-cyclic structure is obtained from NMR studies.

NMR Studies of Adduct I. Adduct I was purified and cleaved enzymatically to the level of riboflavin as described under Experimental Procedures. The 270-MHz FT NMR spectrum was recorded. Results are shown in Figure 5. In oxidized riboflavin the 7,8-CH_3 resonances occur as two distinct singlets at 2.25 and 2.42 ppm, respectively, each containing three protons (Masatsune & Yoshimasa, 1972). In adduct I the 7,8-CH_3 resonances are observed as a single peak at 2.28 ppm. This upfield shift and merging of the two methyl resonances are consistent with NMR spectra of reduced flavins and results from an increased electron density within the isoalloxazine ring system (Ghisla et al., 1976). This peak is therefore assumed to contain six protons and is used as a standard to determine integrations. The broad multiplet at 1.97 ppm integrates to two protons. These protons are coupled to one or more protons at 3.65 ppm because upon irradiation at 3.65 ppm the broad multiplet at 1.97 ppm narrows to a quintet. The ribityl protons appear as a group of indistinguishable peaks between 3.5 and 3.8 ppm. This region integrates to 8.7 protons, indicating that one or more cyclopropanol-derived protons are present in this region.

To simplify the NMR spectrum, 1-[^{2}H]cyclopropanol was synthesized (as described under Experimental Procedures) and used to inactivate methanol oxidase. The covalent adduct formed between 1-[^{2}H]cyclopropanol and flavin cofactor was isolated, purified, cleaved to the riboflavin level, and examined by NMR. Spectral differences are immediately apparent in the ribityl region between 3.5 and 3.8 ppm. This region now integrates to 7.2 protons as compared to 8.7 protons for fully protonated adduct I. The peak at 3.65 ppm decreases dramatically in relation to its neighbors in deuterated as compared to protonated adduct I. Proton decoupling experiments were performed, and no coupling was observed between resonances at 1.97 and 3.65 ppm. Downfield of this region the multiplet between 4.31 and 4.34 ppm integrating to 2.1 protons remains unchanged as a result of deuteration. The results of this isotopic substitution experiment indicate that the proton originating at the C-1 position of cyclopropanol has a chemical shift of 3.65 ppm in adduct I. This chemical shift is consistent with that expected for a carbon bonded to a hydroxyl function.

These NMR results, in accord with previous data, indicate that adduct I is an N_5,C_{4a}-cyclic structure (structure A, Chart I). The β-methylene protons in structure A would be expected as a multiplet between 1.8 and 2.0 ppm, slightly downshifted from the position (1.6 ppm) of the corresponding methylene protons in N_5,C_{4a}-propano-bridged 4a,5-dihydroflavin (Ghisla et al., 1976) due to their position β to a hydroxyl function. A multiplet at 1.97 ppm is observed. The observed coupling between protons at 1.97 and 3.65 ppm is consistent with the assignment of the peak at 3.65 ppm to a proton α to an hydroxyl function and β to a methylene group as in this structure. Model compounds indicate that in N_5-alkyl-substituted flavins the methylene protons adjacent to N_5 appear between 3.4 and 4.2 ppm (Ghisla et al., 1973). Although integrations indicate that three non-ribityl protons are likely in this region, their

Chart I: Possible Structures for Adduct I, Adduct III, and Model Flavin Compounds[a]



[a] When referring to flavin adducts, C_α is defined as the cyclopropanol-derived carbon with bound oxygen (originally C_1 of cyclopropanol), C_β is defined as the cyclopropanol-derived carbon β to this position, and C_γ is defined as the cyclopropanol-derived carbon γ to this position.

identification is obscured by the presence of complex ribityl resonances.

Structure A of Chart I is also consistent with data already cited; acid and base lability, pK_a at 10.5, and an absorption peak at 342 nm. Furthermore, it would be expected to ring open to form a three-carbon aldehydic species covalently linked to flavin at N_5 (structure B, Chart I). An equilibrium between the ring structure and the aldehydic form could account for the two species detected on HPLC.

An alternative structure which could be considered is one in which C_α is bonded to N_5 as in structure C (Chart I). Although the C_β protons in this structure would be observed close to 1.8 ppm which is consistent with experimental results, the chemical shift of the C_γ protons would be between 1.8 and 2.1 ppm as a result of their position adjacent to the asymmetric C_{4a} carbon of the isoalloxazine ring. This is clearly seen in N_5,C_{4a}-propano-bridged 4a,5-dihydroflavin where the chemical shift of the C_γ protons is 2.19 ppm (Ghisla et al., 1976). In the NMR spectrum of adduct I no peaks are seen in this region. Also, the hydroxylic proton at C_α would be shifted significantly downfield from its usual position. In a relevant model compound (structure D, Chart I), the chemical shift of a proton adjacent to both a hydroxyl function and N_5 of the 4a,5-dihydroisoalloxazine ring is observed at 5.75 ppm (Gartner et al., 1976). Since no comparable resonance is observed in the NMR spectrum of adduct I, structure C, Chart I, can be eliminated.

Purification and Characterization of $NaBH_4$-Reduced Flavin Adduct Species. To obtain a more stable species for further structural study, adduct I was modified by $NaBH_4$ reduction at pH 9.0. The covalent adduct formed between flavin and cyclopropanol was reduced while still enzyme bound. Methanol oxidase (17 μM) was inactivated as previously described with 1-[^{3}H]cyclopropanol (100 μM). Inactivation was allowed to proceed for 60 min (enzyme > 96% inactive). The pH of the reaction mixture was then adjusted to pH 9.0 with 1 M KOH, and $NaBH_4$ reduction was carried out as described under Experimental Procedures. Upon completion, the mixture was applied to Sephadex G-25. In contrast to chromatography results with nonreduced adduct, one major radiolabeled peak containing ≥90% of the applied radioactive material was observed. It comigrates with the small molecule fraction. This material was purified by HPLC (system B). Two sharp peaks of radioactivity eluted at 10.0 and 13.8 min, respectively. The material eluting at 10.0 min (referred to as adduct II) was found to be unstable. In contrast, the material with a retention time of 13.8 min (referred to as adduct III) is very stable and contains close to 66% of the radioactivity applied to the column. For these reasons adduct III was chosen for further structural studies.

Purified adduct III has absorbance maxima at 326, 263, 234, and 213 nm. Its extinction coefficient at 326 nm is approximately 9.0×10^3 M^{-1} cm^{-1}. It does not exhibit any pK_a values between 0.5 and 12.0. Adduct III is stable to hydrolysis in both 1 M HCl and 1 M NaOH for at least 12 h as evidenced by identical spectra and HPLC retention times before and after treatment. Its UV spectrum in 6 N HCl exhibits a 4-nm shift toward longer wavelength. The N_{10}-ribityldiphosphoadenosine side chain of adduct III can be sequentially cleaved by phosphodiesterase I, alkaline phosphatase, and sodium periodate without loss of radioactivity. Phosphodiesterase I cleavage results in a modified compound, adduct III′, which has an absorption maximum of 323 nm and an HPLC retention time (system B) of 21.5 min.

It has been shown that partial degradation of riboflavins in certain anaerobic bacteria yield 6,7-dimethyl-N_1-ribityl-2,3-dioxotetrahydroquinoxaline plus urea and CO_2 (Miles et al., 1959). The spectrum of the quinoxaline is nearly identical with that of adduct III′ [λ_{max} 323 nm (ϵ 10700), λ_{max} 263 nm (ϵ 3190), λ_{max} 234 nm (ϵ 12000), λ_{max} 213 nm (ϵ 46000)]. Treatment of both compounds with sodium periodate yields products with λ_{max} at 319 nm. Both are stable to 1 N KOH and 1 N HCl. Consequently, structure E (Chart I) is proposed for adduct III′.

Further evidence for the proposed quinoxaline structure (structure E, Chart I) is provided by the IR spectrum of adduct III′. N_5-Substituted 1,5-dihydroflavins exhibit two strong carbonyl resonances at 1630 and 1690 cm^{-1} corresponding to the C-2 and C-4 carbonyl groups, respectively (Ghisla et al., 1976). Two distinct carbonyl frequencies are also observed in 4a,5-dihydroflavins (1670 and 1720 cm^{-1}) (Ghisla et al., 1976) and tetrahydroflavins (1701 and 1661 cm^{-1}) (Dudley et al., 1967). 2,3-Dioxoquinoxalines, on the other hand, have a single carbonyl absorption between 1660 and 1690 cm^{-1} (Cheeseman et al., 1961). The IR spectrum of adduct III′ shows a single strong carbonyl absorption at 1664 cm^{-1}.

NMR Studies of Adduct III′. The NMR spectrum of adduct III′ is shown in Figure 6 along with difference spectra obtained upon decoupling at 1.98, 3.70, and 4.30 ppm. The aromatic protons at C_6 and C_9 are observed as sharp singlets at 7.38 and 7.33 ppm, respectively. The 7,8-CH_3 protons appear as a singlet integrating to six protons which is shifted slightly downfield (2.32 ppm) from the methyl proton resonance of adduct I. Difference spectra indicate that the multiplet having a chemical shift of 1.98 ppm is coupled both to protons that have a chemical shift of 4.30 ppm and to protons that have a chemical shift of 3.70 ppm. As indicated

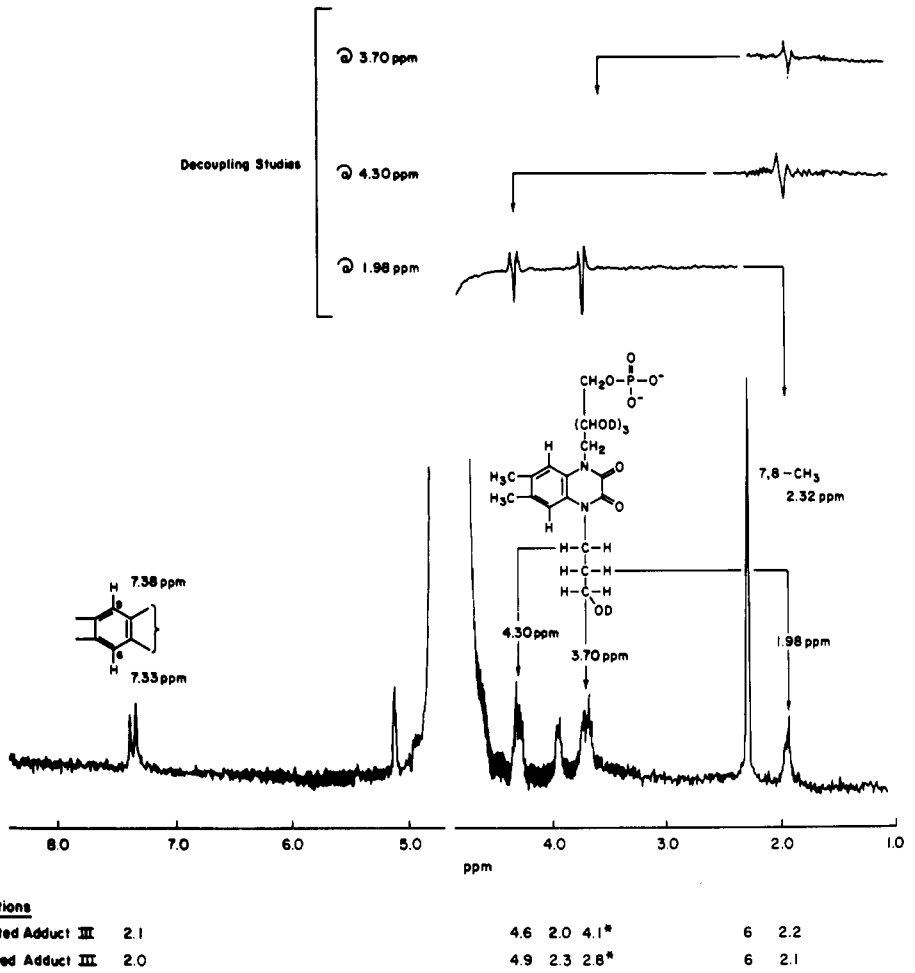


FIGURE 6: 270-MHz FT NMR spectrum of adduct III'. NMR samples were prepared as described under Experimental Procedures. Chemical shift data, integrations, and decoupling studies are discussed in text. Integration values are the average of three separate NMR experiments.

in Figure 6, this multiplet is assigned to the methylene protons at C_β in the proposed structure based upon chemical shift and coupling patterns.

Experiments with adduct III' isolated from 1-[^{2}H]cyclopropanol-inactivated enzyme indicate that the proton originating at the C_1 position of cyclopropanol appears at 3.63 ppm in reduced adduct. Irradiation studies with deuterated adduct III' show coupling between proton(s) at 3.63 and 1.98 ppm, indicating that a proton is bound to C_α. This is strong evidence for the presence of an open chain alcohol. The multiplet appearing at 4.30 ppm and integrating to two protons is coupled to protons at 1.98 ppm but not to protons at 3.63 ppm. This downfield shift compared to that of normal methylene protons, and the coupling pattern, strongly supports their identification as protons attached to a methylene carbon bound to N_5 of flavin. It is concluded that a methylene carbon originating from cyclopropanol is covalently bound to flavin at N_5.

Reconstitution of Methanol Oxidase with FAD and 5-Deazaflavin. Methanol oxidase from *H. polymorpha* has been reconstituted with both commercial FAD and 5-deazaflavin (subsequently referred to as dFAD in the text). Enzyme reconstituted with FAD has an average of 46% methanol oxidizing activity relative to native enzyme. It exhibits absorbance peaks in the visible region with λ_{max} at 370 and 450 nm. These results are in agreement with a previous report (Mincey et al., 1980). Incubation of FAD-reconstituted enzyme (1.2 μM) with cyclopropanol (9.6 μM) results in complete inactivation. Inactivation is irreversible and time dependent and results in the formation of a covalent adduct identical with that formed upon cyclopropanol inactivation of native enzyme.

Table I: Reconstitution of Methanol Oxidase Apoprotein with FAD and 5-Deaza-FAD[a]

	activity	% control activity
control (nondenatured methanol oxidase)	8.00	100
urea/KBr-denatured methanol oxidase without reconstitution with cofactor	0.03	0.4
urea/KBr-denatured methanol oxidase reconstituted with FAD	2.50	31
urea/KBr-denatured methanol oxidase reconstituted with 5-deaza-FAD	0.0	0

[a] Apo-methanol oxidase (20.0 nmol) was prepared and reconstituted with FAD or dFAD according to the procedure described under Experimental Procedures. Upon completion of dialysis, all samples were centrifuged as described and diluted to 1.8 mL with 0.02 M sodium pyrophosphate/0.01 M KCl buffer, pH 8.6. The activity of each sample was assayed by methanol-dependent O_2 consumption and is expressed here as micromoles of O_2 consumed per minute per milligram of protein. The activity of each sample is compared to the activity of the nondenatured methanol oxidase control.

In contrast, enzyme reconstituted with dFAD exhibits UV–visible absorption peaks at 334 and 398 nm. It exhibits no methanol oxidizing activity as measured by O_2 consumption (Table I). Incubation of dFAD-reconstituted enzyme (1.2 μM) with methanol (60 mM) for up to 8 h under anaerobic conditions does not cause any spectral change, indicating that methanol oxidation and concomitant 5-deazaflavin cofactor reduction do not occur. This is in contrast to results with FAD-reconstituted enzyme where incubation with 6 mM

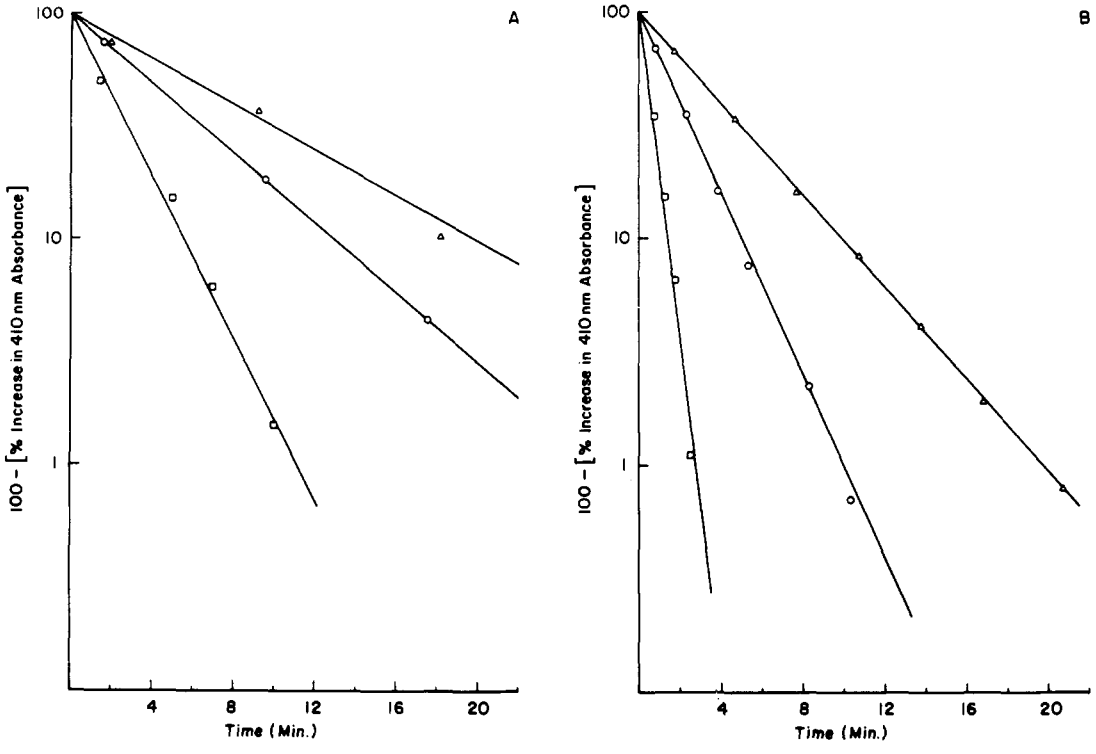


FIGURE 7: (A) Kinetics of oxidation of enzyme-bound $dFADH_2$ by HCHO. A 1.0-mL sample of dFAD-reconstituted methanol oxidase (1.2 μM) prepared as described under Experimental Procedures was transferred to an anaerobic cuvette. The solution was made anaerobic by flushing with argon for 30 min. Enzyme-bound dFAD was reduced by anaerobic titration with sodium dithionite as described under Experimental Procedures. HCHO was then added anaerobically to final concentrations of 0.85 (△), 1.7 (○), and 3.4 (□) mM, and the resulting changes in absorbance at 410 nm were recorded as a function of time. (B) Kinetics of oxidation of free $dFADH_2$ by HCHO. A 1.0-mL sample of dFAD (9.6 μM) was transferred to an anaerobic cuvette and the solution made anaerobic by flushing with argon for 30 min. dFAD was reduced by anaerobic titration with sodium dithionite. HCHO was then added anaerobically to final concentrations of 3.3 (△), 6.6 (○), and 20.0 (□) mM, and the resulting changes in absorbance at 410 nm were recorded as a function of time.

methanol causes an immediate (<0.5 min) decrease in absorbance at 450 nm.

The enzymatically derived reduction potential for the dFAD–$dFADH_2$ couple is −0.312 V (Fisher & Walsh, 1974), significantly more negative than the −0.205-V (Draper & Ingraham, 1968) reduction potential of the FAD–$FADH_2$ couple. Since the reduction potential of the acetaldehyde–ethanol couple equals −0.197 V, and the formaldehyde–methanol couple equals −0.190 V (Latimer, 1964), it is clear that the preferred equilibrium lies toward reduced substrate and oxidized dFAD. This has been observed experimentally for a number of dFAD-reconstituted enzymes (Hersh & Jorns, 1975; Walsh et al., 1976). Experiments were performed to determine if reduced dFAD-reconstituted methanol oxidase could catalyze the reduction of HCHO to CH_3OH with the concomitent reoxidation of $dFADH_2$ to dFAD. All experiments were carried out under strictly anaerobic conditions. Formaldehyde was added to enzyme reconstituted with $dFADH_2$, and the change in absorbance at 410 nm was recorded. As can be seen from Figure 7B, reoxidation is a pseudo-first-order process dependent upon formaldehyde concentration. The second-order rate constant for reoxidation was 2.19 M^{-1} s^{-1}. No saturation was observed at 13.3 mM formaldehyde. Similar results were observed with acetaldehyde; the second-order rate constant is 0.10 M^{-1} s^{-1}. The rate constant for reaction with glyceraldehyde is 0.39 M^{-1} s^{-1}.

The reaction of aldehydes with free $dFADH_2$ (nonenzyme bound) was also determined (Figure 7B). Second-order rate constants for HCHO and CH_3CHO were 1.39 M^{-1} s^{-1} and 0.05 M^{-1} s^{-1}, respectively.

The rate of reaction of enzyme-bound $dFADH_2$ with acetaldehyde and formaldehyde is approximately twice as fast as that with free $dFADH_2$. This is a small rate acceleration, and we believe that the reaction with enzyme-bound $dFADH_2$ is not enzyme catalyzed. The slight rate acceleration seen in the reoxidation of enzyme bound $dFADH_2$ as compared to free $dFADH_2$ could result from a stabilization of the anionic form of $dFADH_2$ by the enzyme. Further evidence against enzyme catalysis is provided by the lack of saturation in the reaction with formaldehyde and by the observation that glyceraldehyde, which is not a substrate for methanol oxidase, is reduced.

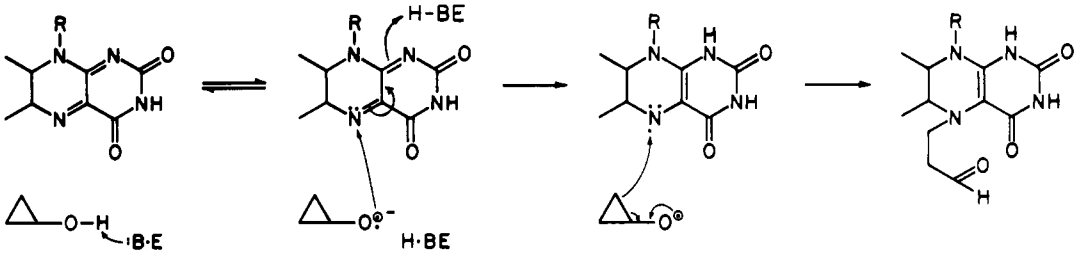
Evidence for Reversible Adduct Formation between dFAD-Reconstituted Enzyme and Cyclopropanol. Incubation of dFAD-reconstituted methanol oxidase (1.2 μM) with 1-[^{3}H]cyclopropanol (200 μM) results in a decrease in absorbance at 398 nm similar to that which occurs upon dFAD reduction with NaS_2O_4 or $NaBH_4$. The initial enzyme solution contained 9.4 nmol of oxidized dFAD. Of this dFAD, 1.3 nmol appeared to be reduced by the addition of 1-[^{3}H]-cyclopropanol (equivalent to 13.8% total dFAD).[1] The cyclopropanol-treated enzyme was denatured and protein precipitated as described under Experimental Procedures. The resulting supernatant fluid, which contained tritiated material, was chromatographed by reverse-phase HPLC (system B). All radioactivity eluted at the solvent front (10.3 min) as does cyclopropanol. A stable covalent adduct between cyclopropanol and dFAD, if formed, would be expected to have a retention time closer to that of dFAD itself (26.3 min) due to the dominating effects of the 5-deazaisoalloxazine ring with its attached N_{10}-ribityldiphosphadenosine side chain.

These results suggest that incubation of cyclopropanol with dFAD-reconstituted enzyme results in the formation of a re-

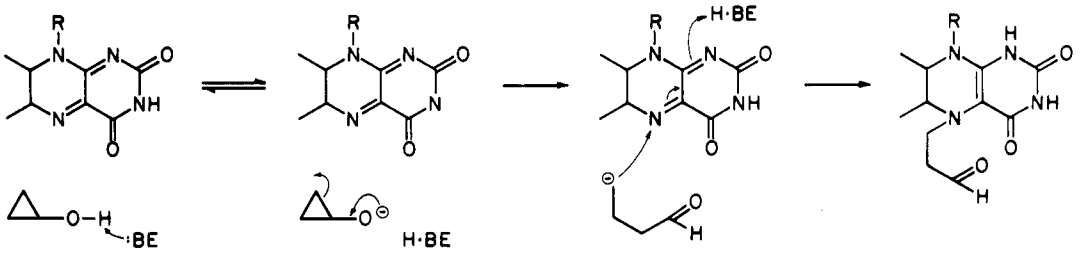
[1] In native enzyme 28% of FAD cofactor is reduced by the addition of excess cyclopropanol.

Scheme I: Possible Mechanisms of Cyclopropyl Ring Cleavage

a) Radical-Induced Ring Cleavage-



b) Base-Catalyzed Ring Cleavage-



versible addition compound between cyclopropanol and dFAD. An experiment was, therefore, carried out to verify this possibility. dFAD-reconstituted enzyme (1.2 μM) was incubated with 1-[^{3}H]cyclopropanol (9.6 μM) for 60 min at 25 °C and then passed through a Sephadex G-25 column (6 cm × 0.5 cm) equilibrated with sodium pyrophosphate buffer (pH 8.6); 0.4-mL fractions were collected. Each fraction was assayed for radioactivity and UV absorbance at 280 nm. The amount of radioactivity coeluting with protein was equal to 0.79 mol of cyclopropanol/mol of dFAD reduced by the addition of 1-[^{3}H]cyclopropanol (determined spectrally). The protein was precipitated with 1% perchloric acid at 25 °C. After neutralization with potassium bicarbonate, this material was chromatographed by reverse-phase HPLC (system A). The only radioactive peak observed had a retention time of 13.6 min and comigrated with cold carrier cyclopropanol. In this experiment only 31% of the original radioactivity was recovered.

Reaction of Methanol Oxidase with Cyclopropylcarbinol. The action of methanol oxidase on cyclopropylcarbinol was examined to determine whether ring-opened products were formed. Formation of such products would be indicative of the involvement of cyclopropylcarbinol radicals in catalysis (MacInnes et al., 1982). Cyclopropylcarbinol was found to be a substrate for methanol oxidase as measured by rate of oxygen consumption. The V_{max} equals 8.8 μmol/min^{-1} (mg of enzyme)$^{-1}$ in air-saturated buffer, and K_m equals 149 mM.

The products formed from the oxidation of cyclopropylcarbinol were identified. A 0.4-mL sample of methanol oxidase (0.6 μM) was incubated in the presence of 250 mM cyclopropylcarbinol for 30 min at 25 °C as described under Experimental Procedures. Protein was removed by precipitation with perchloric acid, and the concentration of aldehyde present in the supernatant fluid was assayed to be 12.6 mM. The aldehyde-containing supernatant fluid was chromatographed on a Bio-Rad Organic Acids column (system D), and two peaks were observed. The first, eluting at 20.0 min, corresponded to excess cyclopropylcarbinol. The second peak, eluting at 26.0 min, contained a product formed through the action of methanol oxidase on cyclopropylcarbinol. This material was not present in reaction mixtures stopped at time zero. The material eluting at 26.0 min was collected and reacted with 2,4-dinitrophenylhydrazine. The resulting hydrazone was washed, filtered, and redissolved in $CDCl_3$, and its NMR spectrum was recorded. The spectrum was that expected for the 2,4-dinitrophenylhydrazone of cyclopropanecarboxaldehyde. This result indicates that the major product of the reaction of methanol oxidase with cyclopropylcarbinol is cyclopropanecarboxaldehyde.

In a parallel experiment, the aldehyde-containing supernatant fluid collected as above was reacted with 2,4-dinitrophenylhydrazine, and the corresponding hydrazones were examined by paper chromatography (system F) and reverse-phase HPLC (system H). Again, the only product observed was cyclopropanecarboxaldehyde. These data indicate that no detectable ring-opened product is formed during the oxidation of cyclopropylcarbinol by methanol oxidase.

Discussion

Cyclopropanol irreversibly inactivates methanol oxidase in a time-dependent manner. Inactivation is accompanied by covalent modification of the flavin cofactor as evidenced by a decrease in 450-nm absorbance and the subsequent isolation from inactivated enzyme of an adduct containing approximately 1 mol of cyclopropanol/mol of active flavin. Structural studies with isolated adduct show that an N_5,C_{4a}-cyclic species is formed [(A), Chart I]. Formation of the adduct involves opening of the cyclopropyl ring and covalent bond formation between one of the methylene carbons of cyclopropanol and N_5 of the flavin. These data suggest that the effectiveness of cyclopropanol as a suicide inactivator of methanol oxidase is, in part, dependent upon cyclopropyl ring strain facilitating ring cleavage and the proximity of the ring-opened species to the electron-deficient N_5 position of the flavin cofactor.

Two mechanisms can be envisioned for adduct formation between cyclopropanol and the flavin cofactor of methanol oxidase. The adduct could be formed through a radical mechanism (Scheme Ia) in which the initial step is interaction of the flavin cofactor and cyclopropanol to form Fl· and a cyclopropoxy radical. The latter ring opens to a 3-propanal radical which then combines with Fl· to form the covalent adduct in a concerted process. Ring-opening reactions of cyclopropoxy radicals are reported to be very rapid (DePuy et al., 1972). Although a rate constant for radical-induced

ring opening of cyclopropanol is not available, the cyclopropoxy radical is such a transient species that attempts to generate it by formation of the cyclopropyl nitrite followed by photolysis (DePuy et al., 1972) resulted only in the detection of the ring-opened radical.

Alternatively, the adduct could be formed through an ionic mechanism (Scheme Ib) initiated by proton abstraction from the hydroxyl group followed by ring opening. The carbanion, formed as a result of ring opening, would be oriented at the active site in such a way that attack at N_5 is favored. Ring opening and adduct formation probably occur in a concerted process. This mechanism is analogous to a nonenzymatic reaction involving 1-phenylcyclopropanol (Thibblin & Jencks, 1979). In that reaction, the carbanion reacts with a proton provided by H_2O or a protonated nitrogen base. In the enzymatic reaction the electrophilic N_5 takes the place of the proton.

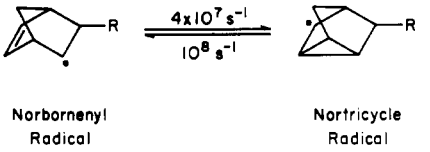
Evidence in favor of the radical mechanism is provided by the reaction of cyclopropanol with apo-methanol oxidase reconstituted with dFAD. In contrast to the reaction of native methanol oxidase, apo-methanol oxidase reconstituted with dFAD does not form an irreversible covalent adduct with cyclopropanol. This lack of reaction suggests that adduct formation involves an intermediate semiquinone, i.e., a radical process. The 5-deazaflavin semiquinone is a high energy species and is not likely to participate as a reaction intermediate; hence, adduct formation does not occur.

Although irreversible covalent adduct formation involving cyclopropyl ring cleavage does not occur, a reversible addition complex was formed that dissociated upon protein denaturation to regenerate cyclopropanol and dFAD. The structure of the adduct was not definitely established. However, it is likely that the adduct is formed by addition of the cyclopropoxide anion to C_5 of dFAD. It is noteworthy that in nonenzymatic model reactions between aldehydes and dihydroflavins an adduct has been detected in which an N_5 carbinolamine adduct is formed (Bruice, 1975). Kinetic studies by Bruice indicate that this reaction does not lie on the catalytic pathway for aldehyde reduction.

It is of interest to compare the rates of ring opening for the nonenzymatic ionic reaction and for the reaction of cyclopropanol with methanol oxidase. For the ring-opening reaction which occurs in the base-catalyzed conversion of cyclopropanol to propionaldehyde $k = 1.5 \times 10^{-4}$ s^{-1}.[2] For the inactivation of methanol oxidase by cyclopropanol $k_{cat} = 4.0 \times 10^{-1}$ s^{-1}. The rate constant for ring opening of the enzyme-bound cyclopropanol must be equal or larger. The rate of ring opening in the enzymic reaction is, therefore, at least 2.7×10^3 times faster than for the nonenzymatic ionic reaction. The factor of 2.7×10^3 underestimates this difference since the rate constant for the ionic reaction applies to the cyclopropoxide ion ($pK_a = 14.4$),[2] and it is unlikely that the enzyme-bound cyclopropanol is totally ionized. We believe that the rapid rate of ring opening which occurs in the enzymatic reaction argues against the ionic mechanism and in favor of the radical mechanism. The possibility that the rapid rate of ring opening in the enzymic reaction is due to strong electrophilic participation by N_5 of the flavin cannot be excluded though.

[2] The first-order rate constant for the breakdown of the cyclopropoxide anion, k, was measured by modification of the procedure of Thibblin & Jencks (1979) in that disappearance of cyclopropanol was monitored by reverse-phase HPLC (system A). The pK_a of cyclopropanol was obtained from the slope of a plot of $1/k_{obsd}$ vs. $1/[OH^-]$ for the base-catalyzed cleavage of cyclopropanol according to the equation derived by Thibblin & Jencks (1979).

Scheme II: Radical-Induced Cyclopropyl Ring Formation and Cleavage in the Sterically Hindered Norbornyl System



Methanol oxidase reconstituted with dFAD does not catalyze the oxidation of methanol nor does enzyme reconstituted with reduced dFAD catalyze the reduction of formaldehyde (the slight rate enhancement observed for the reduction of HCHO by $dFAD_2$-reconstituted enzyme has been discussed in the text, and for the reasons indicated we feel that it is not enzyme catalyzed). This lack of reaction is expected if radical intermediates are involved. In contrast, enzymes thought to catalyze initial carbanion formation followed by hydride transfer (Ghisla et al., 1984) or two-electron transfer (Fischer et al., 1976; Jorns & Hersh, 1976) exhibit substrate oxidizing activity when reconstituted with 5-deazaflavin.

In summary, the data for methanol oxidase are consistent with a radical mechanism, although such a mechanism has not been established. A radical mechanism is in accord with the conclusion reached from model studies that the oxidation of alcohols by flavins occurs through a radical process (Bruice, 1975).

We have shown that the oxidation of cyclopropylcarbinol by methanol oxidase leads exclusively to the formation of cyclopropanecarboxaldehyde. Similarly, cyclopropylmethylamine is converted exclusively to cyclopropanecarboxaldehyde by mitochondrial monoamine oxidase.[3]

The oxidation of cyclopropylcarbinol by various enzymes has been used as a criterion for radical intermediates. It is known from nonenzymatic reactions that cyclopropylcarbinyl radicals undergo a rapid ring-opening reaction with $k = 1.3 \times 10^8$ s^{-1} (Maillard et al., 1976). It has, therefore, been argued that formation of products in enzymic reactions in which the cyclopropyl ring is no longer intact provides evidence for a radical mechanism, and failure to find such products establishes that a radical mechanism is not involved (MacInnes et al., 1982). For instance, the oxidation of cyclopropylcarbinol by alcohol dehydrogenase leads to the formation of cyclopropanecarboxaldehyde. No evidence of ring-opened products was detected. It was concluded that this establishes that reactions catalyzed by alcohol dehydrogenase do not involve radical intermediates (MacInnes et al., 1982). We do not agree with this argument, although the conclusion in this instance may be correct. We believe that failure to find ring-opened products does not exclude radical processes for the following reason. The alkyl radical generated upon ring opening of a cyclopropylcarbinyl radical in solution can undergo a number of reactions; it can abstract H· from the solvent, it can dimerize, or it can undergo electron-transfer reactions. The analogous radical formed at the active site of an enzyme may not have these options and, furthermore, may be sterically restricted so that it will readily undergo ring closure. It has been established that radical-induced cyclopropyl ring cleavage reactions in a restricted system are readily reversible (Scheme II; Ingold, 1973). It is, therefore, possible that at an enzyme active site a cyclopropylcarbinyl radical forms and ring opens. The resulting radical is sterically hindered and cannot undergo reactions that quench the radical. Therefore, ring closure will occur rapidly. Eventually, oxi-

[3] B. Sherry, unpublished results.

dation will occur at the carbinol carbon, cyclopropanecarboxaldehyde will be formed, and no ring-opened products will be detected.

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Registry No. Adduct I, 96021-00-0; adduct III, 95999-22-7; dFAD, 57818-88-9; methanol oxidase, 56379-53-4; cyclopropanol, 16545-68-9.

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