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Presence of a flavin semiquinone in methanol oxidase

(radicals/oxidations)

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ABSTRACT **Methanol oxidase from *Hansenula polymorpha* contains five "red" flavin semiquinones and two oxidized flavins per octamer. Addition of substrate results in the reduction of the two oxidized flavins but does not affect the flavin semiquinones. Enhanced water proton relaxation rates indicate that the unpaired electron of the flavin semiquinones is accessible to the solvent and this accessibility is significantly decreased upon binding of the suicide inhibitor cyclopropanol. In the native enzyme, the semiquinones are not oxidizable by air. All flavins were resolved from the enzyme, and holoenzyme was reconstituted by addition of oxidized flavin. The reconstituted enzyme was catalytically active. The specific activity was 50% that of the original enzyme. It was concluded that the semiquinone is not required for the oxidation of methanol, although it may be present at an otherwise intact site.**

A methanol oxidase has been isolated from the yeast *Hansenula polymorpha* DL-1 (1). The enzyme contains FAD and is an octamer with a subunit molecular weight of 83,000 (1). The published spectrum of a similar enzyme attracted our attention because it indicates that a chromophore other than FAD is present (2). The spectrum suggests that the chromophore might be the semiquinone of FAD. We, therefore, undertook experiments to fully characterize the additional chromophore and to identify its role in the catalytic process.

METHODS

Enzyme Isolation and Assay. *H. polymorpha* strain DL-1 was a gift of Cooney. The enzyme was isolated as described (1, 3), and it was homogeneous upon $NaDodSO_4$ gel electrophoresis. Its specific activity was 17 international units/mg. Enzyme activity was determined at 37°C by measuring O_2 consumption in an O_2 electrode. The assay mixture contained 0.1 M potassium phosphate buffer (pH 7.5), 0.3 μM catalase, 6.5 mM methanol, and 0.1 μM enzyme.

Analytical Procedures. All optical spectra were determined with a Perkin Elmer model 559 spectrophotometer. The longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of water protons were measured at 24.3 MHz and 23°C in 40-μl samples with an NMR Specialties PS 60-W pulsed NMR spectrometer. The molar relaxations ($1/T_{1p}[R\cdot]$, $1/T_{2p}[R\cdot]$) were calculated by using the equation

$$1/T_{ip}[R\cdot] = \left(\frac{1}{T_i} - \frac{1}{T_i^o}\right) \Big/ [R\cdot], \qquad i = 1, 2$$

in which $1/T_i$ is the total relaxation rate, $1/T_i^o$ is the diamagnetic control, and [R·] is the concentration of flavin semiquinone determined by double integration of the ESR spectra by using a picrylhydrazyl standard. The diamagnetic controls were the buffer (0.3 M potassium phosphate, pH 7.5) containing the same amount (7.6 mg/ml) of the diamagnetic protein, pyruvate kinase. Approximately half of the effect of methanol oxidase on $1/T_1$ of water protons and 90% of its effect on $1/T_2$ were due to the paramagnetic contributions, resulting in a somewhat larger error in $1/T_{1p}[R\cdot]$ (see Table 2).

Enzyme Resolution and Reconstitution. Enzyme (90 μM) in 125 μl of 30 mM potassium phosphate buffer (pH 7.5) was added to 0.3 ml of a solution containing 3.5 M KBr, 6 M urea, and 30 mM potassium phosphate buffer (pH 7.8) at 25°C. The absorption spectrum was monitored. All subsequent reactions were carried out at 4°C. When all of the flavin semiquinone was converted to oxidized flavin, the reaction mixture was applied to a Sephadex G-25 (fine) column (9 × 1 cm). The column was equilibrated with 30 mM potassium phosphate buffer (pH 7.8) containing 6 M urea. Then 0.25 ml of 2 mM FAD was placed on the column. The FAD band was allowed to move approximately 3 cm from the top of the column. At this point the enzyme was placed on the column, and 0.5-ml fractions were collected. In this procedure the FAD originally present in the enzyme is separated from the enzyme, but the enzyme merges with the FAD band originally applied to the column. The protein-containing fractions were pooled and then dialyzed for 10 hr against 30 mM potassium phosphate buffer (pH 7.5). At the end of this time, the enzyme was dialyzed for 10 hr against 20 mM sodium pyrophosphate buffer, pH 8.6/10 mM KCl. At this point, all unbound FAD was removed. At the end of the dialysis, the enzyme was centrifuged to remove denatured protein. A control experiment was carried out in which FAD was not first placed on the Sephadex column. Under these conditions, the protein contained no FAD and was catalytically inactive.

RESULTS

The spectrum of yeast methanol oxidase in the presence and absence of substrate is shown in Fig. 1. The ratio of absorbances at 375 nm and 460 nm suggests that a flavin semiquinone is present in addition to oxidized flavin. From the spectrum of the oxidized and reduced enzyme, the nature and number of flavin species present were determined. The results are summarized in Table 1. There are five flavin semiquinones per octamer and two oxidized flavins that can be reduced upon substrate addition. Addition of $Na_2S_2O_4$ or of the suicide inactivator cyclopropanol (unpublished results) also results in the reduction of the two oxidized flavins but does not affect the semiquinones. In the native enzyme, the semiquinones are stable in air.

The data calculated from published spectra for the enzyme from *Kloeckera* (2) are shown in Table 1. This enzyme also contains more than four flavin semiquinones per octamer but its oxidized flavin content is higher.

To confirm the presence of a flavin semiquinone, we examined the ESR spectrum of the enzyme from *H. polymorpha* (Fig. 2). The g value was 2.0031 ± 0.001 (SD) with diphenyl-

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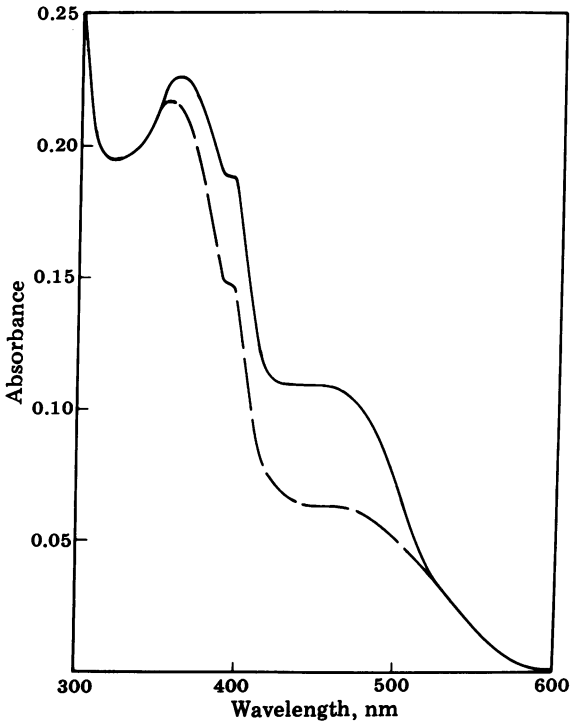

FIG. 1. Optical spectrum of methanol oxidase. The solution contained 20.5 μM enzyme (0.75 mg), 30 mM potassium phosphate buffer (pH 7.5), and 3 mM methanol at 25°C. —, No methanol; - -, with methanol.

picrylhydrazyl [g = 2.00345 (6)] as a standard, and the linewidth was 14 G. The values are characteristic of a red flavin semiquinone. From the peak area with Cu(II) EDTA and diphenylpicrylhydrazyl standards it was determined that 5 ± 0.4 flavin semiquinones are present per octamer, in agreement with the number of semiquinones determined spectroscopically (Table 1). The intensity of the radical signal was only slightly decreased (6–10%) by addition of substrates or cyclopropanol.

Because the ESR spectrum is not significantly affected by substrate, another approach was used to determine whether the substrate interacts with the species containing the flavin semiquinone. The accessibility of the flavin semiquinone to the solvent was determined by measurements of the longitudinal ($1/T_1$) and transverse ($1/T_2$) proton relaxation rates of water

Table 1. Flavin content of methanol oxidase

Flavin	No. of flavins per octamer	
	H. polymorpha	*Kloeckera*
Semiquinone	4.9*	4.2†
Oxidized flavin	2.0‡	4.2†
Total flavin after trichloroacetic acid precipitation	6.8§	—
A_{375}/A_{460}	1.9	1.3†

* Calculated from absorbance at 375 nm and at 460 nm after reduction with substrate. ϵ_{375} = 13.3 × 10^3 liter M^{-1} cm^{-1}; ϵ_{460} = 3.9 × 10^3 liter M^{-1} cm^{-1}. These values are derived from the semiquinone form of D-amino acid oxidase (4).

† Calculated from the data of refs. 2 and 4.

‡ Calculated from ΔA at 460 nm after addition of substrate: ϵ_{460} = 11.3 × 10^3 liter M^{-1} cm^{-1} (5).

§ Enzyme was precipitated by addition of trichloroacetic acid [5% (wt/vol) final concentration]. The FAD content of the supernatant fluid was determined.

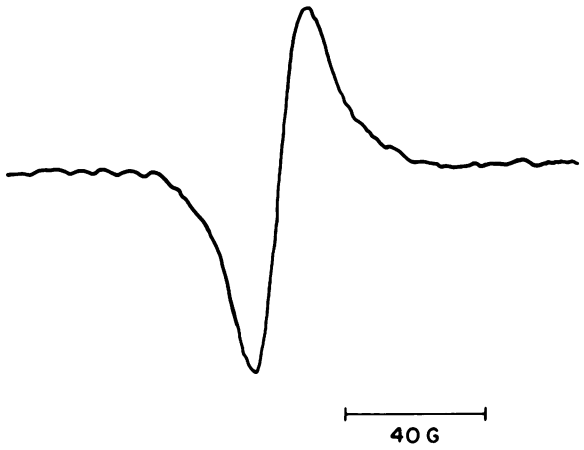

FIG. 2. ESR spectrum of methanol oxidase. The solution contained 90 μM enzyme (7.6 mg/ml) and 30 mM potassium phosphate buffer (pH 7.5). Spectra were taken at −169°C. Spectral width, 400 G; microwave power, 2 mW; microwave frequency, 9.140 GHz; scan time, 4 min; time constant, 1 sec; receiver gain, 5 × 10^3. Concentrations were determined by comparison with Cu(II) EDTA (50 μM).

(Table 2). In comparison with a free flavin radical [10-ethyllumiflavin semiquinone (7)], methanol oxidase caused a 5.5-fold enhancement of the paramagnetic effect of the bound flavin semiquinone on $1/T_1$ of water protons (ϵ_1) and a 240-fold enhancement of the effect on $1/T_2$ of water protons (ϵ_2), indicating that the unpaired spin of the enzyme-bound flavin radical is highly accessible to solvent. The binding of the suicide inactivator cyclopropanol (Table 2) caused a 33% ± 14% decrease in ϵ_1 and a 60% ± 5% decrease in ϵ_2, indicating a significant decrease in the access of water protons to the flavin radical in the enzyme–cyclopropanol complex. These results show that the environment of the semiquinone is affected by the presence of substrate and excludes the possibility that the species containing the semiquinone does not interact with the substrate.

To further examine the role of the semiquinone in the catalytic process, we resolved the enzyme and reconstituted it with oxidized flavin. The reconstituted enzyme contains no spectroscopically detectable semiquinones (<5% of total flavin). It is catalytically active and its specific activity is 37–58% that of the original enzyme. The reconstituted enzyme contains six to eight flavins per octamer. The spectrum of the reconstituted enzyme in the presence and absence of methanol is shown in Fig. 3. The addition of methanol leads to the reduction of two flavins per octamer. This is the same number of flavins that are reduced when substrate is added to the enzyme prior to resolution and reconstitution. On the other hand, addition of $Na_2S_2O_4$ results in the reduction of all enzyme-bound flavins in the reconstituted enzyme.

Table 2. Water proton relaxation by free and enzyme-bound flavin radicals at 24.3 MHz and 23°C

R·	M^{-1} sec × 10^{-3}			
	$1/T_{1p}$[R·]	$1/T_{2p}$[R·]	ϵ_1	ϵ_2
Methanol oxidase	0.730 ± 0.100	32 ± 3	5.5 ± 0.8	241 ± 27
Methanol oxidase–cyclopropanol	0.485 ± 0.080	13 ± 1	3.6 ± 0.6	96 ± 10
10-Ethyllumiflavin semiquinone*	0.130 ± 0.02	0.13 ± 0.02	1.0	1.0

* From ref. 7.

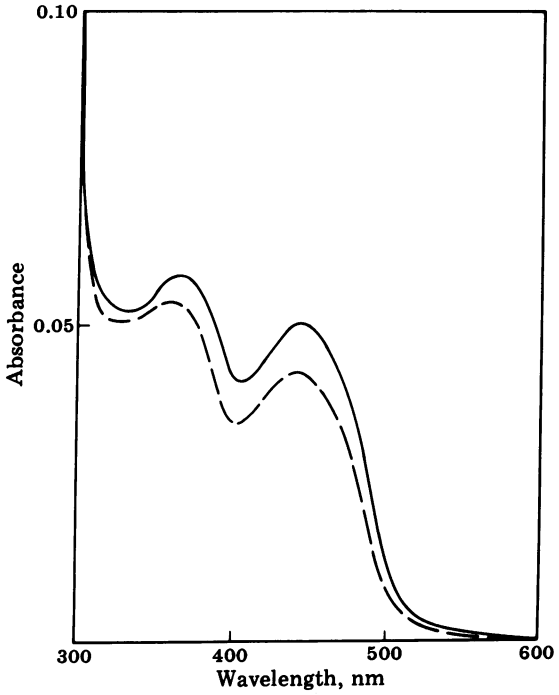

FIG. 3. Optical spectrum of reconstituted methanol oxidase. Apoenzyme was prepared and reconstituted. —, Reconstituted enzyme in 30 mM potassium phosphate buffer (pH 7.5); – –, plus 12.5 mM methanol at 25°C.

No metals could be detected when the enzyme was examined by plasma emission spectrometry† (8).

DISCUSSION

The results show that highly purified methanol oxidase from *H. polymorpha* contains oxygen-stable flavin semiquinones. The octameric enzyme contains five flavin semiquinones and two oxidized flavins. Only the latter are subject to reduction by substrate. The analogous enzyme from *Kloeckera* also appears to contain flavin semiquinones although the ratio of oxidized flavin to flavin semiquinone is higher, as indicated by the ratio A_{375}/A_{460} (Table 1).

The paramagnetic effects of the enzyme-bound flavin radical of methanol oxidase on $1/T_1$ and $1/T_2$ of water protons are 1.5- and 34-fold greater, respectively, than the corresponding effects of flavodoxin semiquinone under similar conditions (7). These differences, especially the large one in $1/T_2$, cannot be due solely to differences in the dipolar correlation times for methanol oxidase and flavodoxin. In general, correlation times for the dipolar effects of enzyme-bound radicals on water protons are dominated by the rapid rotational motion of water molecules weakly hydrogen bonded to the radical and are usually $<10^{-9}$ sec (7, 9). Hence, the greater relaxivity of the flavin radicals of methanol oxidase as compared to flavodoxin results in part from the greater access of solvent protons to the delocalized spin in methanol oxidase. The x-ray structure of flavodoxin indicates that the edge of the dimethyl benzene ring of the flavin is exposed to solvent (10). Because 15% of the unpaired spin density of flavin semiquinones is on the dimethyl benzene ring whereas the remaining 85% of the spin density is on the middle ring (7, 11), it is suggested that the middle ring of the flavin semiquinone may be exposed to solvent in methanol oxidase. The binding of the suicide inactivator cyclopropanol near the reaction center of the flavin (which is on the middle ring) could explain the significant decrease in accessibility of solvent to the semiquinone. Alternatively, the binding of cyclopropanol at a distant catalytic site could induce a conformation change near the flavin radical, decreasing its exposure to solvent.

The amount of semiquinone present is not significantly changed by the presence of substrate or the suicide inactivator, cyclopropanol. Furthermore, the reconstituted form of the enzyme, which contains no flavin semiquinone, is catalytically active. The specific activity of this reconstituted enzyme is approximately 50% that of the original holoenzyme. The flavin semiquinone is, therefore, not required for catalysis, although it may well occupy an otherwise intact catalytic site that can bind cyclopropanol. Because the specific activity of the reconstituted enzyme is lower, it is possible that the presence of the semiquinone may enhance catalytic activity. It is equally likely that the lower specific activity may be the result of partial denaturation during the resolution procedure. At this time, there appears to be no function for the flavin semiquinone. Possibly, in the intact cell, methanol oxidase carries out an additional function that may require the flavin semiquinone.

† Thomas R. Gilbert (Harold E. Edgerton Research Laboratories, New England Aquarium, Boston, MA) performed the metals analysis by plasma emission spectrometry.

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