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Unusual Redox Properties of Electron-Transfer Flavoprotein from *Methylophilus methylotrophus*†

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ABSTRACT: The most positive redox potential ever recorded for a flavin adenine dinucleotide (FAD) containing protein has been measured for an electron-transfer flavoprotein (ETF) synthesized by *Methylophilus methylotrophus*. This potential value, 0.196 V versus the standard hydrogen electrode (vs SHE), was measured at pH 7.0 for the one-electron reduction of fully oxidized ETF (ETF_{ox}) to the red anionic semiquinone form of ETF ($ETF^{\bullet-}$). Quantitative formation of $ETF^{\bullet-}$ was observed. The first successful reduction of ETF from *M. methylotrophus* to its two-electron fully reduced form was also achieved. Although addition of the second electron to $ETF^{\bullet-}$ was extremely slow, the potential value measured for this reduction was −0.197 V vs SHE, suggesting a kinetic rather than thermodynamic barrier to two-electron reduction. These data are believed to be consistent with the postulated catalytic function of ETF to accept one electron from the iron–sulfur cluster of trimethylamine dehydrogenase (TMADH). The second electron reduction appears to have no catalytic function. The very positive potential measured for this ETF and the wide separation of potentials for the two electron reduction steps show that this ETF is a unique and interesting flavoprotein. In addition, this work highlights that while ETFs exhibit similar structural and spectral properties, they display wide variations in redox properties.

Electron-transfer flavoproteins (ETFs)[1] are FAD-containing enzymes that exist as biological electron-transfer links between enzymes of mitochondrial and bacterial degradation pathways and their respective electron-transport chains. As outlined by Davidson et al. (1986), it is of interest to compare and contrast the properties of ETFs from taxonomically diverse sources to ascertain those properties that are conserved among the ETFs and to characterize those properties that render ETF specific for its respective electron donor. The most fundamental properties to be evaluated in such a survey are the oxida-

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[1] Abbreviations: $E^{0\prime}_1$, midpoint potential of first electron reduction; $E^{0\prime}_2$, midpoint potential of second electron reduction; ETF, electron-transfer flavoprotein; ETF_{ox}, oxidized ETF; ETF_{red}, two-electron-reduced ETF; $ETF^{\bullet-}$, anionic, one-electron-reduced ETF; $ETF_{red}H^-$, anionic, hydroquinone form of ETF_{red}; $FADH^-$, anionic form of FAD hydroquinone; FMN^-, anionic, one-electron-reduced form of FMN; $FMNH_2$, FMN hydroquinone; MV^{2+}, oxidized methylviologen; $MV^{\bullet+}$ one-electron-reduced MV; TMADH, trimethylamine dehydrogenase; TMPD, tetramethyl-1,4-phenylenediamine dihydrochloride.

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tion–reduction properties of the ETFs, since electron transport is the essential role that ETFs play.

When the obligate methylotroph *Methylophilus methylotrophus* is grown on trimethylamine as its carbon source, it synthesizes a trimethylamine dehydrogenase (TMADH) plus an ETF whose only known function is to act as the electron acceptor for TMADH. This ETF contains 1 mol of FAD, and its exists as a heterodimer with subunit molecular weights of 38 000 and 42 000 (Davidson et al., 1986). This TMADH–ETF system from *M. methylotrophus* was found to be very similar to that of the bacterium W3A1. In fact, Davidson et al. (1986) observed enzymatic and immunological cross-reactivity between the two systems. Thus, study of the spectroelectrochemical properties of the *M. methylotrophus* ETF can have a major impact on the understanding of electron transfer in both TMADH–ETF systems. Measurements have been made on the redox potentials of TMADH from W3A1 (Barber et al., 1988; C. P. Pace, personal communication). These data together with the crystal structure of TMADH from W3A1 completed to 2.4-Å resolution (Lim et al., 1986), the amino acid sequence, and the spectroelectrochemical data reported here will allow for a more complete understanding of the mechanism of electron transfer between TMADH and ETF.

Davidson et al. (1986) characterized some of the spectral and redox properties of ETF from *M. methylotrophus*. They found that reduction of ETF by a mixture of TMADH and trimethylamine resulted in formation of a nearly quantitative amount of $ETF^{\bullet-}$. Further additions of trimethylamine failed to reduce ETF to its fully reduced form. Likewise, neither dithionite reduction nor photoreduction in the presence of EDTA and 5-deazariboflavin were able to reduce the $ETF^{\bullet-}$. Steenkamp and Gallup (1978) were also able to use dithionite to reduce ETF from W3A1 to its one-electron-reduction form, but further reduction to fully reduced ETF was not observed. In the study reported here, fully reduced ETF from *M. methylotrophus* was obtainable by the spectroelectrochemical method developed by Stankovich (1980, 1983) using electrochemically generated reduced methylviologen as the redox mediator. Previously, this spectroelectrochemical methodology had been successfully applied by Husain et al. (1984) and by Pace and Stankovich (1987) for the measurement of the redox potentials of ETFs from pig liver and *Megasphaera elsdenii*, respectively. The present paper reports the redox properties of ETF from *M. methylotrophus*. The relationship between this ETF and TMADH from W3A1 with respect to these redox properties as well as the differences in redox properties among the various ETFs studied thus far are discussed.

Materials and Methods

Methylviologen and phenosafranine were purchased from British Drug House, Poole, England. The indicator dyes 1,2-naphthoquinone and TMPD were purchased from Aldrich Chemical. Dichlorophenolindophenol, FAD, and phenazine methosulfate were purchased from Sigma Biochemical. Potassium ferricyanide was from J. T. Baker. Indigo disulfonate was purchased from MCB. Pyocyanine was synthesized according to the method of McIlwain (1937). The indicator dye 8-chlororiboflavin was the gift of Dr. J. P. Lambooy, University of Maryland, and 1-deazariboflavin was the gift of Dr. David Graham, Merck Sharpe and Dohme. Lumiflavin-3-acetate was the gift of Dr. Vincent Massey, University of Michigan, and Dr. Sandro Ghisla, University of Konstanz, Konstanz, West Germany. Electron-transfer flavoprotein was purified from *M. methylotrophus* as described by Davidson et al. (1986). When the enzyme was stored a −20 °C for greater than 1 month, an increase in the absorbsance at 410 nm with time was observed (see Results). Therefore, the enzyme was kept in a −80 °C freezer for long-term storage.

The spectroelectrochemical cell methodology was as previously described (Stankovich, 1980; Stankovich & Fox, 1983). Electrochemical experiments were performed with a PAR 173 potentiostat and a PAR 175 coulometer. Apple 2+ or 2E interfaced Cary 210 and Cary 219 spectrophotometers with thermostated cell compartments were used.

All experiments were done anaerobically at 4 °C in 0.05 M KP_i buffer at pH values of 6.4 and 7.0 and in 0.05 M sodium pyrophosphate buffer at pH values of 7.7 and 8.5. All buffers contained 10% (v/v) ethylene glycol.

In an effort to identify the degradation product of ETF, the HPLC method of Negri et al. (1987) was used. Enzyme-bound chromophore(s) were resolved from the apoenzyme by two different methods. One method involved boiling the ETF solution for a few minutes, and the second involved the addition of trichloroacetic acid to a final concentration of 5%. The precipitated apoenzymes were removed by centrifugation. An Epson EQUITY I+ interfaced Spectra-Physics HPLC system composed of an SP 4290 integrator, an SP 8800 pump, and an SP 8450 UV–vis detector was used with a Vydac protein and peptide C_{18} column to effect the separation of possible modified FADs.

Since the ETF as isolated exists as a mixture of ETF_{ox} and $ETF^{\bullet-}$, it was completely oxidized by the following procedure prior to its use in a spectroelectrochemical experiment. An aliquot of thawed ETF was diluted to approximately 2.5 mL. A small amount of solid FAD was added to the solution and mixed. Fifty-microliter aliquots of 1 mM ferricyanide were then added until spectral changes corresponding to excess ferricyanide were observed. The solution was dialyzed overnight against the buffer of choice.

Coulometric experiments were performed in solutions of 15–25 μM ETF in buffer in the presence of 100 μM MV^{2+}. For the determination of the potential of the $ETF_{ox}/ETF^{\bullet-}$ couple, 15–30 μM solutions of ETF were used. The dye 1,2-naphthoquinone was used at pH 6.4 and 7.0 at a concentration of 5 μM, and the dye dichlorophenolindophenol was used at pH 7.7 at a concentration of 1–2 μM. These experiments also contained 1 μM ferricyanide to more accurately determine the initial oxidized spectrum of ETF and also to facilitate potentiometric measurements in the oxidized direction. The potentiometric experiment performed at pH 7.0 also contained 1 μM phenazine methosulfate to facilitate electron mediation. Equilibrium was considered established when the potential drift was less than 0.001 V/10 min.

For the determination of the potential of the $ETF^{\bullet-}/ETF_{red}$ couple, 15–30 μM amounts of ETF were used. The dyes lumiflavin-3-acetate (1 μM) and phenosafranine (1 μM) were used at pH values of 7.0 and 7.7, respectively. At pH 8.5, both phenosafranine (1 μM) and 1-deazariboflavin (1 μM) were used together. Some of these experiments were performed on the same ETF sample used for the measurement of the first electron reduction; others were done separately. For the experiments performed at pH 7.7 and 8.5, the dyes were added under positive nitrogen pressure after the completion of the first electron reduction. Here spectra were taken at points in which the potential drift was less that 0.001 V/5 min.

All potentials are reported versus the standard hydrogen electrode (SHE).

Results

Coulometric Titrations. As reported by Davidson et al. (1986), the molar absorptivity of ETF_{ox} at 438 nm is 12 600

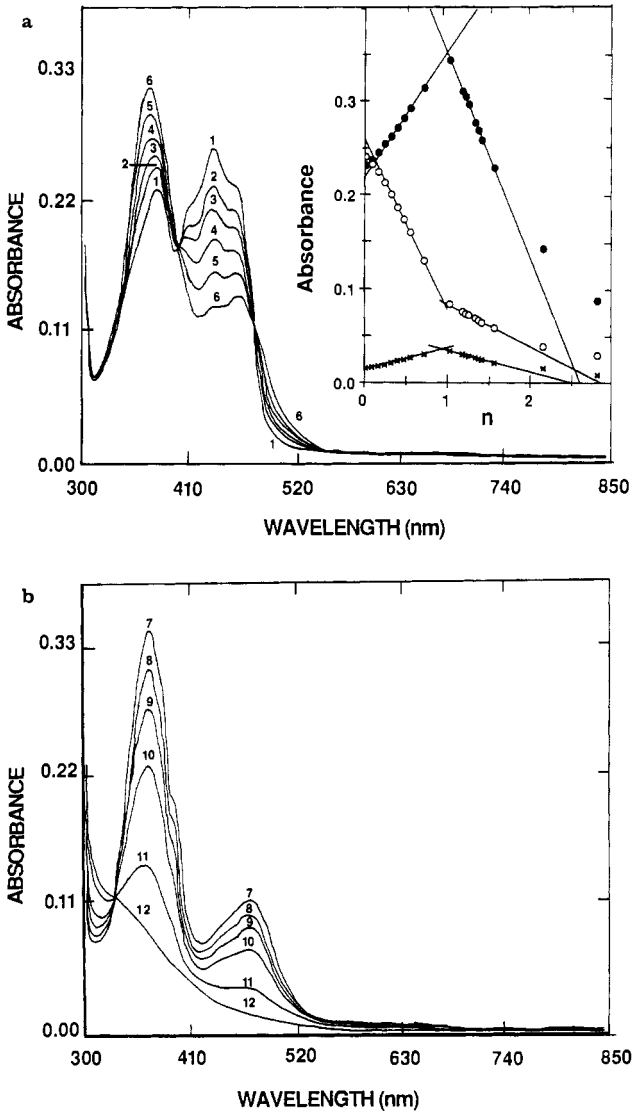

FIGURE 1: Coulometric titration of ETF. Enzyme concentration was 20.83 μM in 0.05 M KP_i buffer, pH 7.0, containing 10% ethylene glycol. Also present was 100 μM MV. (a) Spectrum 1, oxidized ETF; 2, n = 0.1; 3, n = 0.2; 4, n = 0.4; 5, n = 0.5; 6, n = 0.6. (b) Spectrum 7, n = 0.9; 8, n = 1.1; 9, n = 1.2; 10, n = 1.4; 11, n = 1.9; 12, two electron reduced ETF. Inset shows a plot of the absorbance values at 438, 370, and 520 nm versus n, the number of electrons added to the system. (○) 438 nm; (●) 370 nm; (*) 520 nm. Intermediate spectra were removed for clarity.

M^{-1} cm^{-1}. Coulometric titrations of *M. methylotrophus* ETF were performed prior to potentiometric measurements to obtain the molar absorptivities of $ETF^{\bullet-}$ and ETF_{red} and to gain some preliminary data on the spectral and electrochemical behavior of the enzyme. The spectra in Figure 1 follow the reduction of ETF at pH 7.0 to its one-electron-reduced radical form and then to its two-electron fully reduced form. The inset to Figure 1a shows a plot of the absorbances at 438, 370, and 520 nm versus the number of electrons added to the system. The intersection of the two least-squares lines drawn through the data plotted for 370 nm for the first and second electron reductions is considered to be the absorbance at 100% anionic semiquinone formation. This absorbance is used to calculate the molar absorptivities for the anionic semiquinone. The end point of the reduction of ETF is determined by the cessation of spectral changes after several aliquots of added charge. A summary of calculated molar absorptivities is found in Table I, and these molar absorptivities are consistent with those calculated for other flavoproteins (Stankovich & Fox, 1983; Husain et al., 1984). Molar absorptivities calculated for pH 7.0 were used to evaluate the data acquired from experiments performed at other pH values.

Table I: Calculated Molar Absorptivities (M^{-1} cm^{-1}) for the Three Oxidation States of ETF

λ	ETF_{ox}	$ETF^{\bullet-}$	ETF_{red}
438	12 600[a]	4050	1360
370	10 240	16 900	4130

[a] Davidson et al. (1986).

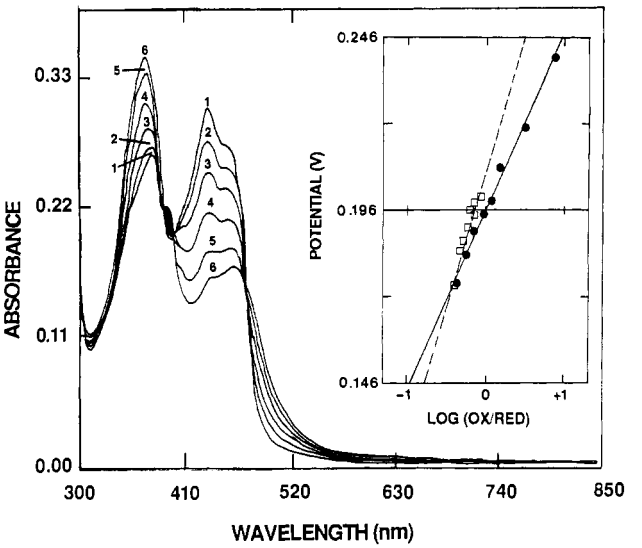

FIGURE 2: Potentiometric titration of ETF_{ox} to ETF_{semi}(first electron). Enzyme concentration was 24.09 μM in 0.05 M KP_i buffer, pH 7.0, containing 10% ethylene glycol. Also present were 100 μM methylviologen, 5 μM 1,2-naphthoquinone, 1 μM ferricyanide, and 1 μM phenazine methosulfate. Spectra are shown for the reductive titration only and are corrected for PMS absorption. Intermediate spectra were omitted for clarity. Spectrum 1, oxidized ETF; 2, E = 0.240 V; 3, E = 0.220 V; 4, E = 0.208 V; 5, E = 0.190 V; 6, E = 0.175 V. Inset shows a Nernst plot of the data: (●) reduced direction, y intercept = 0.196 V, slope = 0.052 V; (□) oxidized direction, y intercept = 0.206 V, slope = 0.077 V.

Equilibration times for the transfer of the first electron to ETF were relatively rapid (10–20 min), while those for the second electron transfer were extremely slow (6–12 h). These phenomena may account for the slight differences in the locations of the line intersections in the inset to Figure 2 (which indicate the transition from the addition of the first electron to the addition of the second electron) among the three wavelengths investigated. Nevertheless, the current efficiency calculated for this titration was 87%, which is considered excellent for an experiment in which long periods of time were required for the transfer of charge from $MV^{\bullet+}$ to $ETF^{\bullet-}$ since some cell oxygen leakage could have occurred. It was not known at this point whether this barrier to the second electron transfer was thermodynamic or kinetic in nature. Measurement of the redox potentials for the reduction of ETF was thus needed to help clarify this unusual behavior.

Potentiometric Titrations. Potentiometric titrations were performed to measure the $E^{0\prime}$ values for the two redox couples of ETF. Initially, a group of redox indicators covering a range of potentials from −0.220 to +0.110 V at pH 7.0 was used in an experiment to approximate the two individual electron redox potential values. In this experiment, phenazine methosulfate (E_m = 0.112 V), pyocyanine (E_m = 0.010 V), indigo disulfonate (E_m = −0.096 V), 8-chlororiboflavin (E_m = −0.115 V), and phenosafranine (E_m = −0.224 V) all titrated after the first electron was transferred to ETF, while the second electron transfer seemed to stabilize in the potential value range −0.150

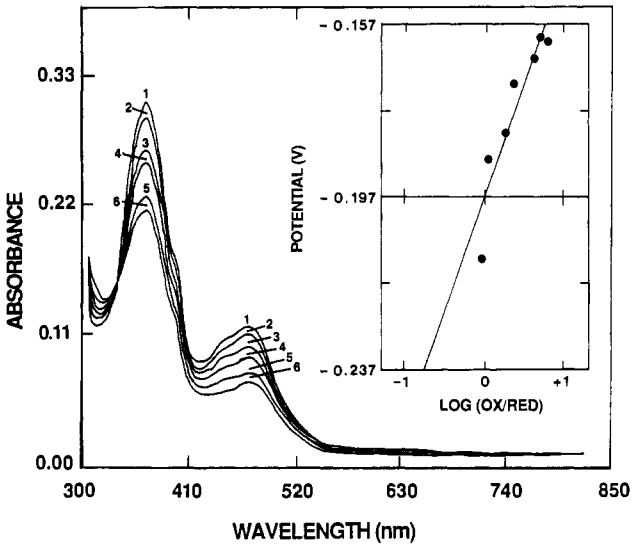

FIGURE 3: Potentiometric titration of ETF_{semi} to ETF_{red}(second electron). Enzyme concentration was 16.97 μM in 0.05 M KP_i buffer, pH 7.0, containing 10% ethylene glycol. Also present were 100 μM MV and 1 μM lumiflavin-3-acetate. Intermediate spectra were omitted for clarity. Spectrum 1, E = −0.161 V; 2, E = −0.165 V; 3, E = −0.171 V; 4, E = −0.182 V; 5, E = −0.188 V; 6, E = −0.211 V. Inset shows a Nernst plot of the data: y intercept = −0.197 V; slope = 0.052 V.

to −0.220 V. This indicated that the potential value sought for the first electron transfer was more positive than those of the dyes and that for a more exact measurement of the second electron potential a dye was needed with a potential value in the range given. In another such experiment at pH 7.0, 4 °C, the dyes TMPD (E_m = 0.290 V), 1,2-naphthoquinone (E_m = 0.177 V), 8-chlororiboflavin, and lumiflavin-3-acetate (E_m = −0.200 V) were used. Here, the two-electron transfers were found to fall in the potential ranges for 1,2-naphthoquinone and lumiflavin-3-acetate, respectively. Since the coulometric titrations and the preliminary potentiometric titrations revealed that the two electrons transfer to the ETF at widely separated redox potentials, it was decided to measure each electron transfer potential separately. These titrations are shown in Figures 2 and 3. The Nernst plots generated for each titration are drawn in the inset of the two figures. For the titration of the first electron, points are plotted from analyses in both the oxidative and reductive directions. The $E^{0\prime}$ value for the $ETF_{ox}/ETF^{\bullet-}$ couple measured in the reductive direction is found to be +0.196 V with a slope of 0.052 V, nearly ideally Nernstian for a one-electron transfer at 4 °C. In the oxidative direction, the $E^{0\prime}$ value is calculated to be +0.206 V, with a slope of +0.077 V. The two measured values are within 0.010 V of each other, indicating the near electrochemical reversibility of the first electron transfer. To date, such a positive potential value has not been measured for any flavoprotein, let alone any ETF, further adding to the catalog of widely varying characteristics displayed by the ETFs from different sources. The potentiometric titration of the $ETF^{\bullet-}/ETF_{red}$ couple was performed only in the reductive direction due to considerations of time (a typical titration took 3–4 days) and, more importantly, due to the nonobservance of semiquinone formation when ETF_{red} is reoxidized. The value measured for this second electron transfer at pH 7.0 was −0.197 V with a nearly ideal Nernstian slope of 0.052 V. This value is not prohibitively negative such that the barrier for the transfer of the second electron is not thermodynamic but rather kinetic in nature. Steric restrictions near the active site may hinder the electron transfer from the reducing agent to $ETF^{\bullet-}$ or perhaps the negative charge of the anionic semiquinone may prevent negatively charged reducing agents, such as dithionite, from approaching the active site.

Table II: Redox Potential Values (V) Measured for ETF as a Function of pH

pH	$E^{0\prime}_1$	$E^{0\prime}_1$ Nernst slope	$E^{0\prime}_2$	$E^{0\prime}_2$ Nernst slope
6.4 (r)[a]	+0.189	+0.038	NA[b]	NA
7.0 (r)	+0.196	+0.052	−0.197	+0.052
7.0 (o)	+0.206	+0.077	NA	NA
7.7 (r)	NA	NA	−0.236	+0.053
7.7 (o)	+0.205	+0.065	NA	NA
8.5 (r)	NA	NA	−0.298	+0.058

[a] (r) denotes points obtained in the reductive direction. (o) denotes points obtained in the oxidative direction. [b] Not available.

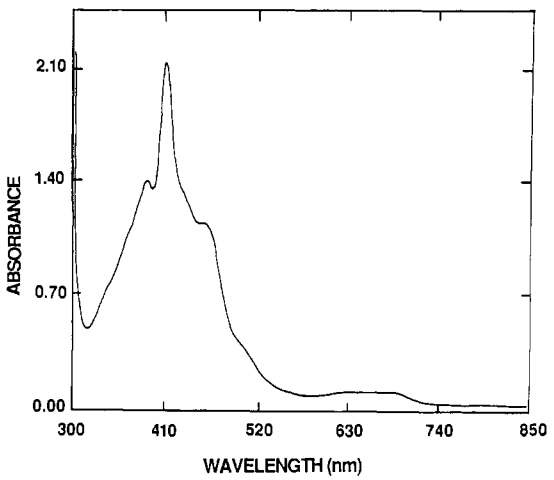

FIGURE 4: Spectrum of ETF after long-term storage at −20 °C.

Redox potential measurements were also made at pH values of 6.4, 7.7, and 8.5 to further characterize the redox properties of the *M. methylotrophus* ETF. The potential–pH behavior is summarized in Table II. The amount of semiquinone thermodynamically stabilized is nearly 100% for each of these experiments (data not shown). The electron potential measured for the $ETF_{ox}/ETF^{\bullet-}$ couple is essentially independent of pH, which is expected whenever anionic semiquinone is quantitatively stabilized. The data for the $ETF^{\bullet-}/ETF_{red}$ couple reveal a −0.060 V/pH unit slope, consistent with the transfer of one proton with one electron to form the $FADH^-$ hydroquinone. Therefore, the data for the first and second electron transfers at pH 7.0 can be summarized according to the thermodynamic relationships shown in eq 1 and 2, respectively.

$$ETF_{ox} + e^- = ETF^{\bullet-}$$
$$E^{0\prime}_1 = +0.196 \text{ V}$$
$$\text{Nernst slope} = 0.052 \text{ V} \quad (1)$$
$$ETF^{\bullet-} + e^- + H^+ = ETF_{red}H^-$$
$$E^{0\prime}_2 = -0.197 \text{ V}$$
$$\text{Nernst slope} = 0.052 \text{ V} \quad (2)$$

Observation of Modified Flavin Formation upon Long-Term Storage of ETF. When ETF was stored at −20 °C for greater than 4 months, the enzyme spectrum changed to that shown in Figure 4. Most experiments performed on ETF from *M. methylotrophus* were performed on enzyme stored in −80 °C conditions such that the amount of modified ETF present in the above experiments is negligible. The chromophore contributing to Figure 4 can be oxidized by ferricyanide, creating a spectrum like that of ordinary FAD. The char-

acteristic 410-nm peak absorbance returns when this same solution is reduced by reduced methylviologen. Further additions of reduced methylviologen reduce the chromophore such that a normal $FAD^{\bullet-}$ spectrum is again observed. Since the oxidized chromophore reduced before what appeared to be the normal oxidized ETF bound FAD when the enzyme existed as a mixture of the two chromophores, interference by this modified ETF in the spectroelectrochemical analyses outlined above would have been observed by an increase and decrease in the absorbance at 410 nm during the course of an experiment. Such changes did not occur. An attempt to separate and analyze these chromophores was undertaken. However, spectra taken after precipitation of the enzyme were not like the spectrum in Figure 4 but rather those of normal FAD. Analysis of this solution by HPLC revealed only one peak, the FAD peak. Therefore, either only one chromophore was resolved from the enzyme or the chromophore contributing to the spectrum in Figure 4 is unstable when removed from the apoenzyme. Efforts to redissolve the colorless protein precipitate by additions of K_2HPO_4, NaOH, and SDS were unsuccessful. The source of these spectral observations will require additional investigation.

Discussion

Both the extremely positive potential value measured for the first electron reduction and the wide separation of the two potential values make ETF from *M. methylotrophus* unique among FAD-containing proteins. The only similarly positive flavoprotein is that of thiamin dehydrogenase, in which $E^{0\prime}_1$ and $E^{0\prime}_2$ were measured at pH 7.2 to be 0.080 and 0.030 V, respectively (Gomez-Moreno et al., 1979). However, this enzyme contains a covalently bound form of FAD, 8α[N-(1)-histidyl]-FAD, which possesses different properties from FAD (Williamson & Edmondson, 1985; Gomez-Moreno et al., 1979; Kenney et al., 1976). Widely separated first and second electron potentials are reported for the flavodoxins from *Azotobacter vinelandii* (pH 8.2, $E^{0\prime}_1$ = 0.050 V, $E^{0\prime}_2$ = −0.495 V), *M. elsdenii* (pH 7, $E^{0\prime}_1$ = −0.115 V, $E^{0\prime}_2$ = −0.372 V), and *Clostridium* MP (pH 7, $E^{0\prime}_1$ = −0.092 V, $E^{0\prime}_2$ = −0.399 V) (Mayhew & Ludwig, 1975). These wide separations of potentials for the flavodoxins have been postulated to be due to conformational changes that are seen in the X-ray crystal structures of flavodoxins when the flavodoxins are reduced (Smith et al., 1983). Perhaps ETF from *M. methylotrophus* possesses a unique chemical structure that can create a highly positive redox potential for its FAD coenzyme in addition to an ability to change the conformation of that structure upon oxidation and reduction of the enzyme such that the enzyme stabilizes FAD in the one-electron-reduced state. The very slow transfer of the second electron and the observation of no radical stabilization upon reoxidation of fully reduced ETF are consistent with this suggestion.

A body of data is finally being developed on the oxidation–reduction properties of the ETFs. The redox properties summarized under Results for the *M. methylotrophus* ETF differ markedly from the redox properties of ETFs from pig liver and *M. elsdenii* (Husain et al., 1984; Pace & Stankovich, 1987). The redox potentials of the first electron reduction ($ETF_{ox}/ETF^{\bullet-}$) and the second electron reduction ($ETF^{\bullet-}/ETF_{red}$) in mitochondrial ETF were measured at pH 7.5 to be +0.004 and −0.050 V. An overall midpoint potential of −0.023 V was calculated with a maximum of 55% anionic semiquinone thermodynamically stabilized. More (nearly 100%) anionic semiquinone is kinetically stabilized when pig liver ETF is reduced by sodium dithionite (Gorelick et al., 1982; Husain & Steenkamp, 1983) and, somewhat like ETF from *M. methylotrophus*, this transfer was relatively sluggish compared to the first electron transfer, requiring 1 h for equilibration. In contrast, ETF from *M. elsdenii* did not thermodynamically stabilize any semiquinone form of FAD at the pH values of 5.5, 7.1, or 8.4 (Pace & Stankovich, 1987). The overall midpoint potentials measured for the two electron reduction at these pHs were −0.185, −0.259, and −0.269 ± 0.013 V, respectively. The redox properties of the three ETFs are presented in Table III.

Table III: Redox Properties of Various ETFs

ETF	$E^{0\prime}$ (V) $ETF_{ox}/ETF^{\bullet-}$	$ETF^{\bullet-}/ETF_{red}$	ETF_{ox}/ETF_{red}	% $ETF^{\bullet-}$ stabilized
M. methylotrophus	0.196	−0.197		100
pig liver[a]	0.004	−0.050	−0.023	55
M. elsdenii[b]			−0.259	0

[a] Husain et al. (1984), pH 7.5. [b] Pace and Stankovich (1987), pH 7.1.

The different redox potential values measured for the three ETFs reflect well on their postulated catalytic functions. In both *M. methylotrophus* and W3A1, the ETFs accept one electron from the Fe_4S_4 cluster of TMADH. The $E^{0\prime}_1$ value measured for ETF in the present study, +0.196 V, is 0.086 and 0.094 V more positive than the values, 0.102 and 0.110 V, measured for the Fe_4S_4 cluster of active and inactive TMADH from W3A1, respectively, by using microcoulometric analysis (Barber et al., 1988) and is 0.121 V more positive than the value, 0.075 V, measured at pH 8.5 for the Fe_4S_4 cluster of inactive TMADH by using the spectroelectrochemical method (Pace, personal communication). These comparisons are consistent with ETF's role as electron acceptor for TMADH. The large separation of potentials between the first and the second electrons for ETF from *M. methylotrophus* strongly suggests that ETF cycles through the $ETF_{ox}/ETF^{\bullet-}$ couple as its only catalytic function. Should any catalytic function be postulated for the second electron transfer, it would necessarily involve an electron donor other than TMADH as the redox potential for $ETF^{\bullet-}/ETF_{red}H^-$ is not thermodynamically accessible for electron transfer to occur from either the flavin ($E^{0\prime}_{FMN/FMN^{\bullet-}}$ = 0.044 V, $E^{0\prime}_{FMN^{\bullet-}/FMNH_2}$ = 0.036 V; Barber et al., 1988) or the Fe_4S_4 cluster of TMADH. Furthermore, this "other species" would have to overcome the kinetic barrier to full ETF reduction through binding or some other as yet uninvestigated interaction that can overcome the very slow electron transfer observed.

Electron-transfer flavoprotein from pig liver is also postulated to cycle between the oxidized and one-electron-reduced form as its primary catalytic mechanism (Gorelick et al., 1985). This conclusion does not appear as clear-cut as with *M. methylotrophus* ETF because only 55% of the pig liver $ETF^{\bullet-}$ is thermodynamically stabilized and the potential for the second electron transfer (−0.050 V) is thermodynamically favorable for electron transfer from several substrate bound acyl CoA dehydrogenases (Stankovich & Soltysik, 1987; Lenn, 1989; Byron, 1989). On the other hand, ETF from *M. elsdenii* is completely involved in two electron transfers as its catalytic function is to accept electrons from the two-electron donor NADH ($E^{0\prime}$ = −0.320 V) and transfer them to a two-electron acceptor, butyryl CoA dehydrogenase ($E^{0\prime}$ = −0.079 V; Fink et al., 1986). The fact that *M. elsdenii* ETF stabilizes no anionic semiquinone and its midpoint potential is much more negative than either *M. methylotrophus* ETF or pig liver ETF is consistent with this level of catalytic function.

Electron-transfer flavoproteins donate their electrons into the electron transport chain of the organism. In *M. methy-*

lotrophus grown on trimethylamine, a comprehensive study on the point in that chain to which ETF donates its electrons has not been undertaken. However, Cross and Anthony (1980) found that when *M. methylotrophus* is grown on methanol, the cytochromes *c* and cytochromes *b* synthesized by these bacteria have remarkably high positive redox potentials (cyt *c*, 0.310–0.375 V; cyt *b*, 0.060–0.225 V). The high redox potentials reported in the present paper are thus consistent with previously studied redox behavior for proteins in the electron transport chain of *M. methylotrophus*.

It is apparent that the ETFs are not as well-defined as a class of flavoproteins as, for example, the flavoprotein acyl CoA dehydrogenases or the flavoprotein oxidases whose redox and spectral properties are quite similar among the proteins belonging to those two classes. However, nature has enabled itself to modify the environment of the FAD in ETFs such that they can cover an incredible range of potential values (+0.200 to −0.260 V) depending on the electron donor with which the ETF interacts. In this paper it was shown that the most positive redox potential value ever measured for an enzyme containing FAD belongs to an ETF. Therefore, it is important that efforts are continued to categorize ETFs and their roles in electron-transfer mechanisms. Electrochemical methods of analysis have been invaluable in this effort to decipher the characteristics of ETFs and other flavoproteins. As more X-ray crystal structures and amino acid sequences of ETFs and other flavoproteins become known, more can be concluded about how such large energy level changes of the electrons in the FAD molecule are executed through binding to apoprotein.

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