Formation of W3A1 Electron-transferring Flavoprotein (ETF) Hydroquinone in the Trimethylamine Dehydrogenase-ETF Protein Complex*

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The electron-transferring flavoprotein (ETF) from Methylophilus methylotrophus (sp. W3A1) exhibits unusual oxidation-reduction properties and can only be reduced to the level of the semiquinone under most circumstances (including turnover with its physiological reductant, trimethylamine dehydrogenase (TMADH), or reaction with strong reducing reagents such as sodium dithionite). In the present study, we demonstrate that ETF can be reduced fully to its hydroquinone form both enzymatically and chemically when it is in complex with TMADH. Quantitative titration of the TMADH-ETF protein complex with sodium dithionite shows that a total of five electrons are taken up by the system, indicating that full reduction of ETF occurs within the complex. The results indicate that the oxidation-reduction properties of ETF are perturbed upon binding to TMADH, a conclusion further supported by the observation of a spectral change upon formation of the TMADH-ETF complex that is due to a change in the environment of the FAD of ETF. The results are discussed in the context of ETF undergoing a conformational change during formation of the TMADH-ETF electron transfer complex, which modulates the spectral and oxidation-reduction properties of ETF such that full reduction of the protein can take place.

Electron-transferring flavoproteins (ETFs) are FAD-containing proteins that serve as important biological electron carriers between enzymes of mitochondrial and bacterial catabolic pathways and their respective respiratory chains, typically passing reducing equivalents to an ETF-ubiquinone oxidoreductase (1). ETFs have been isolated from mammalian systems (human, rat, and pig; Refs. 2–5) as well as bacterial sources, including Paracoccus denitrificans, Megasphaera elsdenii, and Methylophilus methylotrophus (6–8). Both mammalian and bacterial ETFs are αβ heterodimers possessing considerable sequence and structural homology to one another (9, 10). The molecular mass of the α-subunit ranges from 31 to 42 kDa, that of the β-subunit is in the range of 25–38 kDa (1). One equivalent of FAD is noncovalently bound per dimer in all ETFs except the M. elsdenii protein, which binds two FAD molecules per dimer. FAD is the only oxidation-reduction center in these proteins, although the ETFs from human, pig, P. denitrificans, and M. methylotrophus also contain one equivalent of AMP of unknown function (9–12). Unlike most flavoproteins, which function as two-electron carriers, most ETFs are thought to be one-electron carriers, cycling between the oxidized and anionic flavin semiquinone (1). Nevertheless, it has been shown that mammalian ETFs and ETF from P. denitrificans can become fully reduced to the hydroquinone upon dithionite or photochemical reductions, although the reduction from semiquinone to hydroquinone is quite sluggish (4, 6, 13, 14).

ETF from M. methylotrophus W3A1 is the physiological electron acceptor of trimethylamine dehydrogenase (TMADH), an iron-sulfur-containing flavoprotein that catalyzes the oxidative demethylation of trimethylamine (8, 15, 16). The protein is a 65-kDa heterodimer with a 33.7-kDa α-subunit and a 28.9-kDa β-subunit (17); it contains one equivalent each of FAD and AMP per dimer. Unlike mammalian ETFs, which can accept reducing equivalents from a variety of electron donors, including at least nine different dehydrogenases in the mitochondria (18), W3A1 ETF is highly specific for TMADH and cannot be reduced by other dehydrogenases from M. methylotrophus, such as methyamine dehydrogenase and methanol dehydrogenase (8), or by dehydrogenases from other organisms (19). During turnover, W3A1 ETF receives a single reducing equivalent from the 4Fe/4S center of TMADH: the anionic semiquinone/hydroquinone couple of the protein rapidly accumulates in both steady-state (8, 19) and rapid reaction experiments (20).

ETFs are distinct from most other flavoproteins in that the (anionic) semiquinone form is extremely stable (1). In the case of W3A1 ETF, even strong reducing reagents such as dithionite and deazariboflavin radical are unable to reduce the protein to its two-electron reduced hydroquinone form (8, 19). Full reduction of W3A1 ETF has only been achieved by electrochemical means using reduced methyliogen as a redox mediator, with an equilibration time of 6–12 h required for the transfer of the second electron (21). The mid-point reduction potentials obtained for the FAD quinone/semiquinone and semiquinone/hydroquinone couples in this study were 196 mV and −197 mV, respectively. The unexceptional reduction potential for the semiquinone/hydroquinone couple (−197 mV) indicates that the barrier to full reduction of W3A1 ETF is kinetic rather than thermodynamic in nature, as reduction cannot be achieved by either sodium dithionite (−530 mV) or photochemically generated deazariboflavin radical (−650 mV). These reduction potentials indicate, however, that electron transfer from neither the FMN cofactor (E0FMN/0FMNeq = 44 mV; E0FMNag/0FMNeq = 36 mV) nor the 4Fe/4S center (E0 = 100 mV) (22) of TMADH to the ETF semiquinone is thermodynamically favorable.

The above discussion notwithstanding, in examining the re-
action of the TMADH-ETF protein complex with trimethylamine, we have found unexpectedly that \(W_{3A1}\) ETF can be fully reduced to the hydroquinone within the complex. Dithionite titration of the complex indicates that a total of five electrons are taken up, consistent with full reduction of ETF along with TMADH in the complex. Full reduction of \(W_{3A1}\) ETF takes place via the 4Fe/4S center, as reduced phenylhydrazine-inactivated TMADH (in which the enzyme flavin has been covalently modified and rendered redox-inert; Ref. 23) remains capable of reducing ETF from the semiquinone to the hydroquinone state to a substantial degree. The results suggest that the oxidation-reduction properties of ETF are perturbed upon binding to TMADH, and specifically that the apparent kinetic barrier for reduction to the ETF hydroquinone has been overcome in the protein-protein complex.

Electron transfer from TMADH to ETF has been studied previously by following the reaction of reduced TMADH with oxidized ETF, yielding a limiting rate constant of 172 s\(^{-1}\) and a dissociation constant for TMADH\(_{\text{red}}\) and ETF\(_{\text{ox}}\) of 10 mM (20). The reaction of substrate-reduced TMADH with the artificial electron acceptor ferricenium hexafluorophosphate has also been studied (24). The limiting rate constant for electron transfer as a function of temperature was analyzed by electron transfer theory, and an electron tunneling pathway distance of approximately 13 Å was obtained, which correlates with the shortest pathway measured from the 4Fe/4S center to the protein surface of TMADH. This effective electron transfer distance was further refined to 11.3 Å by analyzing the reaction of dithionite reduced phenylhydrazine-inactivated TMADH with ETF; based on the x-ray crystal structure of TMADH and the kinetically determined electron tunneling distance, it was proposed that electrons are transferred to the surface of TMADH at or close to residue Tyr-442 (25). The implication is that ETF docks close to Tyr-442 in forming the protein-protein complex. Kinetic studies of TMADH mutant proteins (Y442F, Y442L, and Y442G) have shown that Tyr-442 indeed plays a significant role in facilitating electron transfer from TMADH to ETF (25).

Given the observations described above, a structural model for the TMADH-ETF complex has been proposed (26). On the basis of this computer-modeled structure, a substantial conformational change of \(W_{3A1}\) ETF during complex formation must take place for efficient electron transfer from the 4Fe/4S center of TMADH to the FAD cofactor of ETF (26). It has also been suggested that such a conformational change alters the environment of the FAD binding pocket and modulates the redox potential of the cofactor. In light of the present work, it appears likely that the proposed conformational change indeed occurs, altering the oxidation-reduction properties of ETF such that its full reduction can take place.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Mono- and dipotassium phosphate, sodium pyrophosphate, trimethylamine hydrochloride (TMA), benzyl viologen, and phenylhydrazine were obtained from Sigma. Sodium dithionite was from Virginia Chemicals. Ferricenium hexafluorophosphate was prepared as described by Lehman and Thorpe (27).

**Protein Purification and Sample Preparation**—**M. methylotrophus** \(W_{3A1}\) was grown on TMA as sole carbon source, and both TMADH and ETF were purified (28). Concentrated protein stock solutions were stored in liquid nitrogen with 15% glycerol as a cryoprotectant. Protein samples for the experiments were prepared in the following way. For TMADH, the thawed, concentrated enzyme stock solution was passed through a Sephadex G-25 column to remove glycerol and the concentration determined from the absorbance at 442 nm of oxidized enzyme (using an extinction coefficient of 27.3 mM\(^{-1}\) cm\(^{-1}\); Ref. 29). In the case of ETF, which is partially reduced as isolated, the protein was reoxidized with excess ferricenium hexafluorophosphate and then passed through a Sephadex G-25 column to remove residual oxidant. The concentration of ETF was determined from the 438-nm absorbance of oxidized protein using an extinction coefficient of 11.3 mM\(^{-1}\) cm\(^{-1}\) (8).

When necessary, protein solutions were concentrated using Amicon Centricon-30 and Centricon-10 concentrators for TMADH and ETF, respectively.

The complex of TMADH with ETF was prepared simply by mixing high concentrations of the two proteins in 1:1 stoichiometry, given the small value expected for the \(K_d\) between the oxidized forms of the two proteins (a \(K_d\) of 10 μM has been determined kinetically for complex formation between reduced TMADH and oxidized ETF; Ref. 20). The complex of TMADH with ETF, which is partially reduced as isolated, the protein was reoxidized with excess ferricenium hexafluorophosphate and then passed through a Sephadex G-25 column to remove residual oxidant. The concentration of ETF was determined from the 438-nm absorbance of oxidized protein using an extinction coefficient of 11.3 mM\(^{-1}\) cm\(^{-1}\) (8).

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dithionite (spectra were continuously taken after adding the reductant until the system reached a new equilibrium; the spectrum of the sample at equilibrium required less than 1 min at the beginning and about 5 min toward the end of titration, and was used for the final plot), and the titration was considered complete when no further spectral change was observed with further addition of the reducing agent. After the titration of the protein complex was complete, the same dithionite solution was then calibrated immediately by titration against a 1-ml solution of anaerobic FMN of known concentration to determine the reductive strength of the sodium dithionite solution. The A_{370} from the titration with the TMADH-ETF complex was then plotted versus the number of electrons added. The experiment was repeated three times, and the total number of electrons taken up obtained were averaged. Since the titration of FMN was performed after the titration of the protein solution, the values obtained are expected to slightly underestimate the electron uptake of the system due to slow oxidation of the dithionite stock solution.

**RESULTS**

**Spectral Changes Observed for the Reaction of the TMADH-ETF Complex with Trimethylamine**—The spectral changes associated with the reaction of the TMADH-ETF complex with TMA are shown in Fig. 1.2 The spectral change observed within 10 s after addition of TMA to the complex (Fig. 1B) is consistent with reduction of the complex by two equivalents of TMA to give the four-electron reduced state, with three electrons required for full reduction of TMADH and one for reducing ETF to the semiquinone form. Subsequently, however, an additional spectral change is observed. This slower spectral change is shown in Fig. 1C and has features, in particular the substantial absorbance decrease at 370 nm reflecting loss of the anionic semiquinone, that suggest further reduction of ETF semiquinone to the hydroquinone state has occurred.

**Dithionite Titration of the TMADH-ETF Complex**—To investigate whether ETF can indeed be reduced fully to the flavin hydroquinone within the TMADH-ETF complex, a quantitative titration with sodium dithionite was performed to determine the total number of reducing equivalents taken up by the protein complex. The absorption spectra of the protein complex in the course of the titration are shown in Fig. 2A. A plot of the absorbance change at 370 nm (following the accumulation and subsequent decay of the anionic semiquinone of both TMADH and ETF) versus the number of reducing equivalents added is shown in Fig. 2B, where it is evident that approximately five electrons are required for full reduction of the complex and that at completion of the titration the absorption at 370 nm, which accumulates transiently in the course of the titration, has been lost, undoubtedly reflecting reduction of the ETF semiquinone to the hydroquinone. The experiment was performed in triplicate, and the average electron uptake of the complex found to be 4.7 ± 0.12 e⁻. Assuming a K_d for binding of the oxidized forms of the two proteins comparable to the value of 10 μM determined kinetically for complex formation between reduced TMADH and oxidized ETF, it is estimated that about 73% of the proteins would form complex under the present experimental conditions. Therefore, a value of 4.7 electrons actually agrees very well with the fact that not all of the protein molecules have formed complex and only those in the complex were able to take up an additional electron. Control experiments were also performed to ensure that the FAD cofactor did not dissociate from ETF in the course of dithionite titration, and this was found not to be the case.3 The results of the dithionite titration clearly demonstrate that the TMADH-ETF complex can indeed take up a total of five electrons and that ETF can be reduced fully to FAD hydroquinone when it is in complex with TMADH.

**Reaction of Three-electron Reduced TMADH (TMADH_{3e⁻}) with ETF Semiquinone (ETF sq)—**To investigate whether full reduction of ETF within the TMADH-ETF complex occurs by

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2 This reaction was necessarily performed under aerobic conditions, as the 1-mm light path cuvette that had to be used (owing to the high concentrations of proteins used) had an open top and it was not technically feasible to make it anaerobic. We note that both reduced TMADH and ETF are unusually stable to air-reoxidation, however, with half-lives for the reaction of approximately 1 h. In particular, it has been shown previously that the reaction of TMADH with TMA (in the absence of ETF) exhibits identical kinetics under aerobic and anaerobic conditions (24, 25). For these reasons, the effect of O_2 on the present reaction is expected to be negligible. That this is the case is evident in the sustained reduction of the enzyme that is observed over the duration of the present experiments. All spectral changes that are observed, even those on the slowest (seconds) time scale, are associated with reduction of the enzyme-ETF complex, and not its reoxidation.

3 After the dithionite titration was complete, the reduced sample was loaded to a small Sephadex G-25 column to determine whether a fraction of free FAD was present. If FAD had dissociated from the protein, it would be rapidly reoxidized by O_2 during gel chromatography and a bright yellow band would appear. The column was also illuminated with UV light to detect any trace amount of FAD, which is highly fluorescent. No yellow fraction or fluorescence was observed for the dithionite-reduced protein sample during the whole process of chromatography, indicating that the FAD cofactor did not dissociate from ETF in the course of dithionite titration.
electron transfer from TMADH rather than direct reduction by sodium dithionite, the following experiment was performed. Equal volumes of 70 μM TMADH3e2 and 70 μM ETFsq were placed into separate compartments of an anaerobic split-cell cuvette and the spectrum recorded. The two solutions were then mixed thoroughly, and the spectrum of the sample was measured every 20 s for 15 min (the reaction was found to go to completion in approximately 10 min). The spectra of the solution before mixing and upon completion of the reaction are shown in Fig. 3A, and the ((15 min after mixing) − (before mixing)) difference spectrum in Fig. 3B. Since the spectra for TMADH3e2, TMADH2e2, ETFsq, and ETFhq are known, the expected spectral change associated with transfer of one electron from TMADH3e2 to ETFsq to give TMADH2e2 (containing FMNhq and oxidized 4Fe/4S center)4 and ETFhq can be calculated, and is shown in Fig. 3C. From a comparison of panels B and C, it is evident that the experimentally obtained difference spectrum agrees well with that calculated from the known spectral properties of TMADH and ETF,5 indicating that electron transfer from TMADH3e2 (presumably via its 4Fe/4S center; see below) to ETFsq was achieved. From the magnitude of the spectral change at 370 nm, it is estimated that electron transfer went to only 30% completion under the present experimental conditions, reflecting an internal equilibrium of the electron distribution between TMADH3e2 and ETFsq before mixing are 70 μM for each protein.

4 At pH 7.0, the electron distribution in two-electron reduced TMADH favors formation of FMNhq and oxidized 4Fe/4S center (31). Therefore, the 4Fe/4S center remains predominantly oxidized after transferring one electron to the FAD of ETF since electron transfer from FMNhq to the 4Fe/4S center is under prototropic control and essentially prevented at pH 7.0.

5 The slight difference seen between the observed and calculated difference spectra is most likely a result of the minor spectral perturbation associated with binding of ETF to TMADH, which is known to occur when the oxidized form of the two proteins bind to each other.

FIG. 2. Dithionite titration of the TMADH-ETF protein complex. A, absorption spectra of the TMADH-ETF protein complex in the course of sodium dithionite titration. B, absorbance of the TMADH-ETF complex at 370 nm versus the number of electrons per complex. The concentrations of TMADH and ETF used in this experiment are 100 μM each.

FIG. 3. Spectral change associated with the reaction of TMADH3e2 with ETFsq. A, absorption spectrum of TMADH3e2 and ETFsq in separate compartments of a split cell cuvette (solid line), and spectrum of the sample after the two solutions were mixed (Δ). B, difference absorption spectrum observed with the reaction of TMADH3e2 with ETFsq ((after mixing) − (before mixing)). C, calculated spectral change associated with one-electron transfer from TMADH3e2 to ETFsq to give TMADH2e2 (containing FMNhq and oxidized 4Fe/4S center) and ETFhq. The concentrations of TMADH3e2 and ETFsq before mixing are 70 μM for each protein.
remains redox-active (23, 27). The spectral change observed upon mixing PHZ-TMADH$_{1e}$ with ETF$_{sq}$ is shown in Fig. 4. The spectra of the protein solution before mixing and 15 min after mixing are shown in Fig. 4A, and spectrum of the sample after the two solutions were mixed (→). B, difference absorption spectrum observed with the reaction of PHZ-TMADH$_{1e}$ with ETF$_{sq}$ (after mixing) − (before mixing). C, calculated spectral change associated with one-electron transfer from PHZ-TMADH$_{1e}$ to ETF$_{sq}$ to give PHZ-TMADH$_{ox}$ and ETF$_{hq}$. The concentrations of PHZ-TMADH$_{1e}$ and ETF$_{sq}$ before mixing are 70 μM for each protein.

![Fig. 4. Spectral change associated with the reaction of reduced phenylhydrazine-inactivated TMADH with ETF$_{sq}$. A, absorption spectrum of reduced phenylhydrazine-inactivated TMADH (PHZ-TMADH$_{1e}$) and ETF$_{sq}$ in separate compartments of a split-cell cuvette (solid line), and spectrum of the sample after the two solutions were mixed (→). B, difference absorption spectrum observed with the reaction of PHZ-TMADH$_{1e}$ with ETF$_{sq}$ (after mixing) − (before mixing). C, calculated spectral change associated with one-electron transfer from PHZ-TMADH$_{1e}$ to ETF$_{sq}$ to give PHZ-TMADH$_{ox}$ and ETF$_{hq}$. The concentrations of PHZ-TMADH$_{1e}$ and ETF$_{sq}$ before mixing are 70 μM for each protein.](http://www.jbc.org/)
consistent with taking a normal unmodified flavin from a more hydrophilic environment into a more hydrophobic one (32–35) and must therefore be due to a perturbation of the environment of the FAD in ETF, rather than the 6-S-cysteinyl FMN of TMADH.

**DISCUSSION**

In the present study, we have demonstrated that full reduction of W$_{SA}$ ETF can be readily achieved both enzymatically (with excess substrate) and chemically (by sodium di-thionite) within the TMADH-ETF protein complex. Transfer of a second reducing equivalent to the ETF semiquinone to give the hydroquinone is mediated by TMADH, as demonstrated by the occurrence of electron transfer upon mixing either TMADH$_{3e^-}$ or PHZ-TMADH$_{3e^-}$ with ETF$_{sq}$ under anaerobic conditions. Previous studies have shown that ETF receives a first reducing equivalent from TMADH exclusively via the 4Fe/4S center in the course of the oxidative half-reaction (20). The fact that reduced phenylhydrazine-inactivated TMADH is also able to transfer an electron to ETF semiquinone for the formation of hydroquinone indicates that the second reducing equivalent is also transferred via the 4Fe/4S center of TMADH, as expected.

The reduction potentials of the redox-active centers in TMADH and ETF have been determined previously. At pH 7.0, the FMN/FMN$_{sq}$, FMN$_{sq}$/FMN$_{hq}$, and Fe$_{red}$/Fe$_{oxid}$ couples of TMADH have potentials of 44, 36, and 110 mV, respectively (22), while the potentials for the FAD/FAD$_{sq}$ and FAD$_{sq}$/FAD$_{hq}$ couples of ETF are 196 and −197 mV (21). The high potential for the FAD/FAD$_{sq}$ couple (196 mV) is consistent with its physiological role in receiving electrons from the 4Fe/4S center of TMADH (110 mV), but that for the FAD$_{sq}$/FAD$_{hq}$ couple in ETF (~197 mV) is substantially lower than those of the centers in TMADH, and further reduction is therefore thermodynamically unfavorable. Nevertheless, the results presented here clearly demonstrate that ETF is able to receive a second reducing equivalent from the 4Fe/4S center of TMADH and become fully reduced when it is in complex with TMADH. This indicates that the relative reduction potentials of the cofactors must be perturbed upon formation of the TMADH-ETF complex.

From the magnitude of the spectral change associated with the reaction of fully reduced TMADH with ETF semiquinone, it is estimated that electron transfer has taken place in about 30% of the TMADH$_{3e^-}$/ETF$_{sq}$ complex to give TMADH$_{2e^-}$/ETF$_{hq}$. Given the extent of electron transfer that is observed, the reduction potential of the FAD$_{sq}$/FAD$_{hq}$ couple of ETF within the complex must be shifted upward by about 3 mV (from that seen in free solution) to a value in the range of 0 mV. So large a change in reduction potential must reflect a substantial change in the environment of the FAD of ETF upon binding to TMADH. On the other hand, electron transfer is found to occur in about 56% of the PHZ-THD$_{sq}$/ETF$_{sq}$ protein complex. Since these percentages reflect the internal equilibrium of electron distribution between the cofactors, the result indicates that the reduction potentials for the cofactors involved (specifically the 4Fe/4S center and FAD cofactor) may be affected to a certain extent by covalent modification of the FMN cofactor in TMADH. According to the Nernst equation, such a difference in equilibrium or electron distribution (30% versus 56%) reflects a moderate ~20-mV difference in relative reduction potentials of the Fe$_{red}$/Fe$_{oxid}$ and FAD$_{sq}$/FAD$_{hq}$ redox couples.

Bearing in mind the uncertainties inherent in the above argument, a set of reduction potentials for the TMADH-ETF complex predicted from the above arguments can be given as: FMN/FMN$_{sq}$, 44 mV; FMN$_{sq}$/FMN$_{hq}$, 36 mV; Fe$_{red}$/Fe$_{oxid}$, 110 mV; FAD/FAD$_{sq}$, 196 mV; FAD$_{sq}$/FAD$_{hq}$, 0 mV. Given this set of potentials, the first reducing equivalent is expected to yield principally the ETF (anionic) semiquinone, with a concomitant increase in absorbance at 370 nm. A second equivalent should go to reduce the Fe/S center of TMADH, although some FMN semiquinone is expected to accumulate. The significant further increase in absorbance at 370 nm that is observed experimentally (Fig. 2) appears greater than might have been expected at this stage of the titration, but it is important to recognize that addition of two reducing equivalents is not likely to generate a homogeneous population of two-electron reduced complex, but rather a distribution consisting principally of one-, two-, and three-electron reduced complex, as has been found to be the case in other complex redox-active systems (see, e.g. Ref. 36). By addition of the third reducing equivalent, however, the absorption increase at 370 nm seen in the course of the reductive titration is at least qualitatively consistent with a system containing ETF essentially completely as the semiquinone, and the FMN of TMADH >33% in the semiquinone form also, with the Fe/S center of TMADH for the most part reduced. Subsequent bleaching of the absorbance at 370 nm as the titration goes to completion clearly indicates the eventual reduction of both the FAD of ETF and the FMN of TMADH to the level of the hydroquinone. It is to be emphasized that this analysis must be regarded as only qualitative, and it is possible that the extent of the transient increase in absorbance at 370 nm observed experimentally reflects some perturbation of the TMADH flavin potentials in addition to that discussed for the ETF SQ/HQ couple, giving rise to a somewhat greater thermodynamic stabilization of the semiquinone of this site than expected on the basis of the reduction potentials for the free enzyme. Future work involving the explicit determination of the reduction potentials of the several sites within the TMADH-ETF complex will address this explicitly. The somewhat qualitative nature of this argument concerning the behavior of the complex in the course of a reductive titration notwithstanding, however, we emphasize that the observation of substantial electron transfer from reduced TMADH to the ETF semiquinone (Figs. 3 and 4) is most readily accounted for by invoking an increase in potential for the ETF SQ/HQ couple of the order of 200 mV.

A three-dimensional structural model for W$_{SA}$ ETF has been constructed using homology modeling techniques (26), based on the crystal structure of human ETF (9). On the basis of this model and the x-ray crystal structure of TMADH (37), it is evident that an electron transfer-efficient complex cannot be formed by the two proteins without a significant structural reorganization. In particular, both the regions surrounding Tyr-442 in TMADH and the FAD of ETF are concave and not physically complementary. It thus has been suggested that ETF undergoes a substantial conformational change upon...
binding to TMADH, with domains I and III rotating ~50° with respect to domain II relative to their initial positions in the structural model (26, 38). Within the protein complex, the conformation adopted by ETF is proposed to be conducive to electron transfer, with the FAD cofactor properly oriented for efficient electron transfer from the 4Fe/4S center of TMADH. In the context of such a structural rearrangement, it is not surprising that the reduction potential of ETF in the electron transfer-active form is perturbed. In addition, the FAD of ETF goes from being exceptionally exposed to solvent to a generally more hydrophobic environment, consistent with the spectral change observed upon binding to TMADH. Our results are thus entirely consistent with the idea that a conformational change in ETF occurs upon binding to TMADH and perturbs the oxidation-reduction properties of ETF, thus facilitating the transfer of a second electron into ETF to allow full reduction of the protein.

In the present work, we have demonstrated that W3A1 ETF can be reduced fully to the flavin hydroquinone form both enzymatically and chemically within the TMADH-ETF complex. This reaction is considerably more facile than is the case with ETF in free solution, taking place on a time scale of minutes rather than hours. It is important to bear in mind that full reduction of the TMADH-ETF complex requires five electrons, and when substrate is used, an intercomplex disproportionation-reduction properties of ETF, thus facilitating the transfer of a second electron into ETF to allow full reduction of the protein.

demonstrates unequivocally that a complex does form and provides a new and convenient experimental probe whereby the protein protein interaction can be monitored.

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