**Electron Transfer and Conformational Change in Complexes of Trimethylamine Dehydrogenase and Electron Transferring Flavoprotein**

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The trimethylamine dehydrogenase-electron transferring flavoprotein (TMADH-ETF) electron transfer complex has been studied by fluorescence and absorption spectroscopies. These studies indicate that a series of conformational changes occur during the assembly of the TMADH-ETF electron transfer complex and that the kinetics of assembly observed with mutant TMADH (Y442F/L/G) or ETF (αR237A) complexes are much slower than are the corresponding rates of electron transfer in these complexes. This suggests that electron transfer does not occur in the thermodynamically most favorable state (which takes too long to form), but that one or more metastable states (which are formed more rapidly) are competent in transferring electrons from TMADH to ETF. Additionally, fluorescence spectroscopy studies of the TMADH-ETF complex indicate that ETF undergoes a stable conformational change (termed structural imprinting) when it interacts transiently with TMADH to form a second, distinct, structural form. The mutant complexes compromise imprinting of ETF, indicating a dependence on the native interactions present in the wild-type complex. The imprinted form of semiquinone ETF exhibits an enhanced rate of electron transfer to the artificial electron acceptor, ferricinium. Overall molecular conformations as probed by small-angle x-ray scattering studies are indistinguishable for imprinted and non-imprinted ETF, suggesting that changes in structure likely involve confined reorganizations within the vicinity of the FAD. Our results indicate a series of conformational events occur during the assembly of the TMADH-ETF electron transfer complex, and that the properties of electron transfer proteins can be affected lastingly by transient interaction with their physiological redox partners. This may have significant implications for our understanding of biological electron transfer reactions in vivo, because ETF encounters TMADH at all times in the cell. Our studies suggest that caution needs to be exercised in extrapolating the properties of in vitro interprotein electron transfer reactions to those occurring in vivo.

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Trimethylamine dehydrogenase (TMADH, EC 1.5.99.7) from the methylotrophic bacterium *Methylophilus methylotrophus* (sp. W3A1) is a homodimeric iron-sulfur flavoprotein that forms an electron transfer complex with electron transferring flavoprotein (ETF (1)). Each subunit of TMADH (molecular mass ~ 83 kDa) contains a covalently linked 6-S-cysteinyl FMN cofactor, a 4Fe-4S center, and a tightly bound ADP of unknown function (2–8). TMADH catalyzes the oxidative demethylation of trimethylamine to form dimethylamine and formaldehyde (Reaction 1),

\[
(CH_3)_3N + H_2O \rightarrow (CH_3)_2NH + CH_3O + 2H^+ + 2e^-
\]  

**REACTION 1**

The mechanism of enzyme reduction by trimethylamine has been addressed by stopped-flow spectroscopy in wild-type (9–11) and mutant (12–15) TMADH enzymes and has recently been reviewed (16). Following substrate reduction of TMADH, electrons are transferred sequentially from the FMN cofactor to the 4Fe-4S center and, subsequently, in an interprotein electron transfer reaction to the FAD of electron transferring flavoprotein (ETF) (17, 18). ETF from *M. methylotrophus* is a heterodimer with subunit masses of 34 and 29 kDa (19). ETF contains a non-covalently bound FAD (1) and AMP of unknown function (20), and it cycles between fully oxidized and semiquinone forms. Full reduction of *M. methylotrophus* ETF to the dihydroquinone form occurs, albeit sluggishly, by electrochemical methods (21) or by dithionite titration in the presence of redox mediators (22). The FAD dihydroquinone is also populated during dithionite titration of the TMADH-ETF complex (23).

Based on the crystal structure of human ETF (24), our modeling studies (25) suggested that the structure of *M. methylotrophus* ETF comprises three domains, arranged in a ‘Y’-shape, with domains I and III forming a shallow bowl (one rigid body) in which domain II (a second rigid body) sits. We have demonstrated using small-angle x-ray scattering methods that domain II is conformationally mobile and that this flexibility produces an ensemble of ETF structures, only a subset of which can dock onto TMADH (26). Recent studies with human ETF have established domain flexibility as a general feature of the ETF family (27), and these dynamic aspects of ETF structure are likely important in optimizing electron transfer rates from redox donor proteins (26, 27).

Using absorption spectroscopy and stopped-flow methods, we demonstrate herein that the time scales of conformational
change in the structure of *M. methylotrophus* ETF on complex formation with the Y442G mutant TMADH enzyme are slower than the observed rates of electron transfer, indicating that in *vitro* electron transfer occurs in metastable TMADH-ETF complexes. We also demonstrate using fluorescence methods that interaction with TMADH elicits a slow conformational change (imprinting) in the structure of ETF. This conformational change is not seen in human ETF incubated with either TMADH or its natural electron donor, medium-chain acyl-CoA dehydrogenase. Our work highlights the complexity of inter-protein electron transfer in the TMADH-ETF system and implications for the mechanism of electron transfer in *vivo* are discussed.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Proteins—Native and wild-type recombinant *M. methylotrophus* ETF were expressed and purified as described by Basran et al. (18) and Jones et al. (26), respectively. αR237A ETF was expressed and purified as described (22). Human ETF was kindly provided by Dr. F. E. Fremr (Department of Pediatrics, University of Colorado School of Medicine). Native and mutant forms of TMADH were expressed and purified as described by Wilson et al. (28) and Basran et al. (18), respectively.

Recombinant human medium-chain acyl-CoA dehydrogenase (MCADH) was expressed from plasmid pTrc MCADH as described (29); however, the isopropyl-1-thio-β-β-galactopyranoside concentration used for induction of protein expression was raised from 0.1 to 1 mM. Harvested cells were resuspended in buffer A (50 mM Tris-HCl, 2 mM EDTA, pH 7.8) and disrupted using a French Press (140 MPa at 4 °C). The extract was clarified by centrifugation at 15,000 × g for 45 min, and solid ammonium sulfate was added to 40% saturation. After centrifugation, the supernatant was applied to a phenyl-Sepharose column previously equilibrated with buffer A containing 1.5 mM ammonium sulfate, and the protein was eluted by a decreasing gradient (1.5 to 0 mM ammonium sulfate contained in buffer A). Fractions were pooled, dialyzed overnight against buffer A, and applied to a Q-Sepharose column previously equilibrated with the same buffer. MCADH was released from the column by gradient elution (0 to 2 M KCl). The pooled fractions were dialyzed against 50 mM potassium phosphate buffer, pH 7.8, and stored (~70 °C) in the presence of 20% ethylene glycol.

**Difference Spectroscopy**—Difference spectroscopy was performed using a Hellma quartz Suprasil Precision Cell, which is a split-cell cuvette containing two compartments, each with a light path of 4.375 mm (total light path including divider is 1 cm). The contents of the separate compartments in the cuvette were mixed by inversion, and spectroscopic analysis of the contents was made before and after mixing to obtain a difference spectrum. Spectral acquisition was carried out in 50 mM potassium phosphate buffer, pH 7.0, at room temperature. For all experiments, concentrations of ETF and TMADH were 15 μM and 20 μM, respectively.

**Fluorimetric Analyses**—Spectrofluorimetric analyses were performed using an SLM Instruments 48000 spectrofluorimeter attached to a 450-watt xenon lamp (except for lifetime measurements, where a 100-watt mercury lamp was used). All analyses were carried out in 50 mM potassium phosphate buffer, pH 7.0, at 20 °C in quartz cuvettes, with a light path of 5 mm. Static emission spectra were measured from 460 to 700 nm, exciting at 450 nm. Static excitation spectra were measured from 300 to 520 nm, measuring emission at 530 nm. All spectra were measured at a protein concentration of 10 μM. Kinetic measurements of ETF fluorescence were carried out using an excitation wavelength of 450 nm and measuring emission at 530 nm. The concentration of ETF used in all experiments was 5 μM, and the concentration of TMADH varied from 0.25 to 15 μM. To minimize the effects of photobleaching on the observed fluorescence signal, measurements were taken at timed intervals commensurate with the time scale of the experiment, and the sample was only exposed to the excitation beam for a maximum of 10 s per measurement.

For anisotropy measurements, GAL-Thompson polarizers were used, both to polarize the excitation light and to polarize the emitted light at both the excitation and emission. For lifetime measurements, a 100-watt mercury arc lamp and an excitation wavelength of 436 nm (corresponding to a peak in the arc lamp emission intensity) were used. Modulation frequencies were generated using an SLM PTS 500 frequency synthesizer. Nanosecond fluorescence lifetimes were calculated from frequency modulation data.

**Solution X-ray Scattering**—Small-angle x-ray scattering (SAXS) data of native and imprinted ETF were collected with the low angle scattering camera on station 2.1 (30) at the Synchrotron Radiation Source, Daresbury, UK, using a position-sensitive multwire proportional counter (31). The sample-to-detector distance of 1.5 m and protein concentrations of approximately 15 mg/ml were chosen to reveal the scattering behavior in the range 0.01 Å⁻¹ ≤ s ≤ 0.08 Å⁻¹ (s is the modulus of the momentum transfer and defined as s = 2 sin θ/λ, where θ is the scattering angle and λ = 1.54 Å is the x-ray wavelength). As established by our previous studies (26, 27), this scattering interval is ideal in directly highlighting any differences in the scattering characteristics of the two protein states as a result of possible domain reorganizations. Further information with regards to data collection, reduction, and analysis have been described previously in detail (26, 27).

**Rapid Kinetic Analyses**—Single-wavelength rapid mixing kinetic experiments were performed using an Applied Photophysics stopped-flow apparatus (SF.17MV), and data were collected and analyzed using an Acom A5000 computer and Spectrakinetics software (Applied Photophysics). Monophasic transients were analyzed using Equation 1,

\[ A_0 = A_1(1 - e^{-kt}) + b \]  

where \( A \) is the absorbance at time \( t \), \( A_0 \) is the initial absorbance, and \( b \) is a floating end point to account for a non-zero baseline. Biphasic transients were analyzed using Equation 2,

\[ A_0 = C_1(1 - e^{-kt_1}) + C_2(1 - e^{-kt_2}) + b \]  

The inorganic redox acceptor ferricenium hexafluorophosphate ([Fe(CN)₆]³⁻) was used in studies of electron transfer from ETF₀ in the imprinted and non-imprinted form. Imprinting of ETF₀ was achieved by mixing wild-type TMADH (1 μM) with ETF₀ (15 μM), and incubating the mixture at 25 °C. Samples of the reaction mixture were analyzed by fluorescence spectroscopy (see “Results”) to ensure that the imprinting reaction proceeded to completion. In all experiments, ETF₉₀ (15 μM, reaction cell concentration) was reacted with ferricenium hexafluorophosphate (concentration range 0.2 to 1 mM, reaction cell concentrations) contained in 50 mM potassium phosphate buffer, pH 7.0. Reactions were performed over the temperature range 5 to 35 °C.

Studies of complex assembly were performed using stopped-flow absorption spectroscopy. ETF (15 μM; in both oxidized and semifluorinated forms) was mixed with oxidized wild-type and mutant (Y442F, Y442L, and Y442G) TMADH enzymes (10 μM). All reactions were performed in 50 mM phosphate buffer, pH 7.0, at 25 °C. Measuring the absorbance change at 482 and 460 nm, respectively, monitored assembly of the TMADH-ETF₂⁻ and TMADH-ETF₉₀ complexes. The rates of absorption change were obtained by approximating first order fitting to the early parts of the reaction transient.

**RESULTS**

**Difference Spectroscopy and Kinetic Studies of Complex Assembly**—We showed previously that assembly of the TMADH-ETF complex could be observed by difference spectroscopy on mixing ETF₀ with TMADHox in a “split” optical cuvette (23). However, we show in this paper that the kinetics of complex formation cannot be measured using the stopped-flow method owing to the fast time scale of the spectral change (Table I). Formation of the ETF₉₀-TMADHox complex can likewise be observed by difference spectroscopy (26), but in this case the absorption changes occur on a time scale that enables characterization by stopped-flow spectroscopy (Table I). Herein, we have extended our work to the Y442F, Y442L, and Y442G TMADH-ETF complexes. The overall spectral changes accompanying complex formation with these mutant enzymes are similar to those observed with the wild-type complex (e.g. Fig. 1), but the kinetics of assembly are impaired (Table I). The spectral changes accompanying the assembly of Y442F and Y442L TMADH with ETF₀ are too rapid to observe using the stopped-flow method but can be observed with ETF₁⁻. The spectral changes accompanying assembly of both the Y442G TMADH-ETF₀ and TMADH-ETF₉₀ complexes are observable in stopped-flow studies (Table I). Furthermore, formation of the Y442G TMADH-ETF₀ and TMADH-ETF₉₀ complexes occurs on much longer time scales than the corresponding rates of electron transfer when normalized to the equivalent concentra-
Conformational Change in Interprotein Electron Transfer

TABLE I

| Enzyme          | Rate constant for complex assembly<sup>a</sup> | Rate constant for electron transfer<sup>b</sup> | Kinetically determined dissociation constant<sup>b</sup> |
|-----------------|----------------------------------------------|-----------------------------------------------|--------------------------------------------------------|
|                 | $k_{on}$ $s^{-1}$ | $k_{off}$ $s^{-1}$ | $k_{cat}$ | $k_{d}$ M<sup>-1</sup>s<sup>-1</sup> |
| Wild-type       | 9.3 × 10<sup>-2</sup> | 1.44 NA | NA |
| Y442F           | 8.4 × 10<sup>-3</sup> | 1.76 49.6 | 28.3 |
| Y442L           | 3.1 × 10<sup>-3</sup> | 1.18 29.0 | 24.6 |
| Y442G           | 2.8 × 10<sup>-3</sup> | 0.14 6.3 | 45.9 |

<sup>a</sup> Observed rates of complex formation are for reactions of TMADH and ETF at concentrations of 5 and 7.5 μM, respectively. Data fitting was performed as described under “Experimental Procedures.”

<sup>b</sup> Data taken from Ref. 18. The electron transfer rate in the wild-type complex displays a linear dependence on ETF concentration, owing to the reaction being limited by the bimolecular association of the component proteins. In the mutant complexes a hyperbolic dependence is observed, owing to a rate-limiting conformational change subsequent to formation of the initial encounter complex. For further details see Ref. 18.

Fluorescence Properties of the Electron Transfer Complex and Its Components—The excitation spectrum of FAD in ETF<sub>ox</sub> coincides with that of free FAD (Fig. 2A). Little, if any, Trp or Tyr contribution was observed at 280 nm in the excitation spectrum for ETF-bound FAD, revealing the absence of resonant energy transfer from Trp and Tyr to FAD. The quantum yield of FAD emission in ETF<sub>ox</sub> and ETF<sub>sq</sub> is, respectively, about 10–15% and 20–30% that of free FAD. A quenching group reduces the FAD emission in ETF, because the intensity of the flavin emission increases very slowly, commensurate with the slow release of FAD from the protein (Fig. 2B). Dynamic deactivation of excited FAD by the adenosine moiety is well known for FAD in aqueous solution (32). However, the crystal structures of human (24) and Paracoccus denitrificans (33) ETF, and the model of M. methylotrophus ETF (25), indicate the adenosine moiety does not contact the isoalloxazine ring in ETF-bound FAD. The quenching group is therefore derived from the protein and not the FAD cofactor itself. The fluorescence lifetime of FAD in ETF<sub>ox</sub> (4.5 ± 0.2 ns) is about twice that for free FAD in water (2.5 ± 0.3 ns; Table II). The corresponding value for ETF<sub>sq</sub> is 2.9 ± 0.2 ns (Table II).

We also investigated the fluorescence properties of the 6-S-cysteinyl FMN in TMADH. The quantum yield of fluorescence emission at 540 nm (excitation 450 nm) for TMADH preparations is very small (–0.04%), and fluorescence from the 6-S-cysteinyl FMN is probably quenched as a result of the proximity of the flavin to the 4Fe-4S center. The lifetime of fluorescence is ~6 ns, compared with 5 ns for free FMN in water. The very low quantum yield, and the long fluorescence lifetime, of TMADH preparations suggest that the emission at 540 nm may not be attributable to the 6-S-cysteinyl FMN in native TMADH; the origin of this fluorescence emission is uncertain, but possibilities include minor contaminating flavoproteins or a small population of non-native TMADH. The very low quantum yield of fluorescence emission for preparations of TMADH has allowed us to investigate flavin fluorescence changes associated with ETF on interaction with TMADH.

<sup>2</sup> The second order rate constant for electron transfer in the Y442F mutant complex is greater than that for the wild-type complex at low (<15 μM) ETF concentrations. However, the rate of electron transfer in the mutant complex approaches a limiting value owing to the hyperbolic dependence of the rate on ETF concentration. In the wild-type complex, the rate of electron transfer is linearly dependent on ETF concentration. The observed rates of electron transfer are therefore substantially greater in the wild-type complex from 15 μM ETF and upwards. For further details see Fig. 3 in Ref. 18.

![Image](Fig. 1. Static difference spectrum and kinetics of assembly for the Y442G TMADH-ETF<sub>ox</sub> and Y442G TMADH-ETF<sub>sq</sub> complex. A, difference spectrum (spectrum after mixing minus spectrum before mixing) for Y442G TMADH (20 μM) and ETF<sub>ox</sub> (30 μM). Inset, kinetic transient at 482 nm showing the absorbance change on mixing of ETF<sub>ox</sub> with Y442G TMADH; data fitting performed as described under “Experimental Procedures.” B, difference spectrum (spectrum after mixing minus spectrum before mixing) for Y442G TMADH (20 μM) and ETF<sub>sq</sub> (30 μM). Inset, kinetic transient at 469 nm showing the absorbance change on mixing of ETF<sub>sq</sub> with Y442G TMADH; data fitting performed as described under “Experimental Procedures.” Conditions: 50 mM potassium phosphate buffer, pH 7.0, 25 °C.)
Incubation of ETF\textsubscript{sq} and ETF\textsubscript{ox} with TMADH leads to a gradual ~4-fold increase in ETF flavin fluorescence, with a half-life of about 30 min and reaching a plateau after about 2 h (Fig. 3A). No similar change in the flavin fluorescence was observed for preparations of either (i) oxidized or reduced TMADH alone, or (ii) ETF alone. The rate of increase in fluorescence was analyzed by approximating first order fitting to the early parts of the progress curves (the reaction is strictly second order). Rates for the development of enhanced fluorescence were found to be linearly dependent on the concentration of TMADH, which is as expected for a structural change in ETF catalyzed by TMADH (Fig. 3B). The absorption spectrum of the FAD following prolonged incubation (>2 h) of ETF\textsubscript{sq} with TMADH is characteristic of the anionic semiquinone (Fig. 4), which, coupled with the magnitude of the fluorescence enhancement, rules out the slow release of FAD from protein (an obvious possibility) as an explanation for the fluorescence enhancement; reduced flavins rapidly re-oxidize (half-life < 1 s) in free solution. However, there is a small increase in the absorption at 450 nm and small decrease at 360 nm (Fig. 4), which could suggest partial oxidation of the ETF-bound FAD. The structurally altered ETF spectrum was, however, resistant to further reduction following addition of trimethylamine to the imprinting mixture, suggesting partial oxidation of the ETF-bound FAD.

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**TABLE II**

| Sample       | Anisotropy, $\tau$ | Polarization, $P$ | Lifetime, $\tau$ | Rotation correlation time, $\theta$ |
|--------------|---------------------|-------------------|------------------|-----------------------------------|
| ETF\textsubscript{sq} | 0.162 ± 0.0005      | 0.225 ± 0.0006   | 4.5 ± 0.2        | 3.1                               |
| ETF\textsubscript{ox} | 0.222 ± 0.0005      | 0.299 ± 0.0006   | 2.9 ± 0.2        | 3.6                               |
| Imprinted\textsuperscript{a} ETF\textsubscript{sq} | 0.116 ± 0.0003      | 0.165 ± 0.0003   | 5.5 ± 0.3        | 2.2                               |
| Imprinted\textsuperscript{a} ETF\textsubscript{ox} | 0.163 ± 0.0005      | 0.227 ± 0.0007   | 3.6 ± 0.2        | 2.5                               |
| Imprinted\textsuperscript{b} ETF\textsubscript{sq} | 0.113 ± 0.0005      | 0.161 ± 0.0006   | ND\textsuperscript{a} | ND\textsuperscript{a} |
| Imprinted\textsuperscript{b} ETF\textsubscript{ox} | 0.163 ± 0.0005      | 0.227 ± 0.0007   | ND\textsuperscript{a} | ND\textsuperscript{a} |

\textsuperscript{a} ETF imprinted with TMADH in the following reaction: ETF (5 \textmu M) + TMADH (5 \textmu M), 50 mM potassium phosphate buffer, pH 7.0; incubated at 20 °C for 2 h.

\textsuperscript{b} ETF imprinted with TMADH in the following reaction: ETF (5 \textmu M) + TMADH (0.25 \textmu M), 50 mM potassium phosphate buffer, pH 7.0, incubated at 20 °C for 48 h.

\textsuperscript{ns} ND, not determined.

Incubation of ETF\textsubscript{sq} and ETF\textsubscript{ox} with TMADH\textsubscript{a} leads to a gradual ~4-fold increase in ETF flavin fluorescence, with a half-life of about 30 min and reaching a plateau after about 2 h (Fig. 3A). No similar change in the flavin fluorescence was observed for preparations of either (i) oxidized or reduced TMADH alone, or (ii) ETF alone. The rate of increase in fluorescence was analyzed by approximating first order fitting to the early parts of the progress curves (the reaction is strictly second order). Rates for the development of enhanced fluorescence were found to be linearly dependent on the concentration of TMADH, which is as expected for a structural change in ETF catalyzed by TMADH (Fig. 3B). The absorption spectrum of the FAD following prolonged incubation (>2 h) of ETF\textsubscript{sq} with TMADH is characteristic of the anionic semiquinone (Fig. 4), which, coupled with the magnitude of the fluorescence enhancement, rules out the slow release of FAD from protein (an obvious possibility) as an explanation for the fluorescence enhancement; reduced flavins rapidly re-oxidize (half-life < 1 s) in free solution. However, there is a small increase in the absorption at 450 nm and small decrease at 360 nm (Fig. 4), which could suggest partial oxidation of the ETF-bound FAD. The structurally altered ETF spectrum was, however, resistant to further reduction following addition of trimethylamine to the imprinting mixture, suggesting partial oxidation of the ETF-bound FAD had not occurred. The fluorescence data thus indicate that TMADH catalyzes a structural change in ETF to yield an altered form of the protein that we term “imprinted ETF.”

Following imprinting of ETF\textsubscript{sq} by TMADH, ETF-bound FAD is readily reduced by inclusion of catalytic amounts of TMADH and the substrate trimethylamine, thus indicating that imprinted oxidized ETF is catalytically competent. Imprinting of ETF is clearly mediated by the interaction of ETF with TMADH during formation of the electron transfer complex. The imprinting reaction is substantially compromised (~10- to 20-fold reduction in the rate) with the Y442 TMADH mutant enzymes and the aR237A mutant ETF (Fig. 5). An interaction between Tyr-442 (TMADH) and aArg-237 (ETF) has been im-
Table II). Calculated values for the rotational correlation time $\tau_F$ for the FAD cofactor in both ETF and imprinted ETF were performed on SAXS data adhering to those reported earlier for native ETF (26).

SAXS parameters for both states of ETF that can be deduced from the SAXS data adhere to those reported earlier for native ETF (26). As a result of this outcome, structural changes as a result of the imprinting reaction. The SAXS data adhering to those reported earlier for native ETF (26)

Within the statistical noise the two curves can be considered to be identical. As a result of this outcome, structural parameters for both states of ETF that can be deduced from the SAXS data adhere to those reported earlier for native ETF (26). The close resemblance of the two scattering profiles is further highlighted as intensity ratio with respect to native ETF (Fig. 6, inset). Within the statistical noise the two curves can be considered to be identical. As a result of this outcome, structural parameters for both states of ETF that can be deduced from the SAXS data adhere to those reported earlier for native ETF (26).

Fluorescence anisotropy and lifetime measurements for the FAD cofactor in both ETF and imprinted ETF were performed (Table II). Calculated values for the rotational correlation time ($\theta$) of the fluorophore were obtained from the Perrin Equation (Equation 3),

$$r = \frac{r_0}{1 + \langle\phi\theta\rangle}$$

(Eq. 3)

where $r$ is the measured anisotropy, $r_0$ is the fundamental anisotropy, and $\tau$ is the fluorescence lifetime. The data (Table II) indicate that the anisotropy values for imprinted ETF$_{sq}$ and ETF$_{ox}$ are less than the corresponding values for non-imprinted samples. The major effect accounting for the reduction in anisotropy is a decrease in the rotation correlation time (Table II); small increases in fluorescence emission lifetime are also apparent, but these effects are small. The FAD fluorophore in structurally imprinted ETF therefore undergoes a larger angular displacement between absorption and emission of a photon than in non-imprinted ETF, suggesting “looser” binding of the fluorophore in imprinted ETF.

Electron Transfer Properties of Imprinted ETF—Reactions of ETF$_{sq}$ and imprinted ETF$_{sq}$ with the artificial oxidant ferricinium were performed to gain additional evidence for altered conformation of the protein. Absorption transients for the oxidation of imprinted ETF$_{sq}$ were monospecific and data were analyzed by fitting to Equation 1 (Fig. 7). In contrast, for reactions with ETF$_{sq}$ absorption transients were biphasic, and observed rate constants were determined by fitting to Equation 2. The observed rates for the slow phase of the reaction were approximately 10-fold less than those for the fast phase (Fig. 8B and inset). Studies of the concentration and temperature dependence of the reaction indicated that the rate of oxidation was enhanced with imprinted ETF$_{sq}$ compared with purified ETF$_{sq}$ (Fig. 8, A and B). The altered kinetic parameters provide additional evidence for conformational change following interaction of ETF$_{sq}$ with TMADH.

**DISCUSSION**

Electron Transfer Can Occur in Metastable TMADH-ETF Complexes—We showed previously that spectral perturbation in the visible region is pronounced for complex formation between ETF$_{sq}$ and TMADH$_{ox}$ (26) and that a smaller perturbation in the visible spectrum is also observed for the ETF$_{sq}$-TMADH$_{ox}$ complex (23). For the ETF$_{sq}$-TMADH$_{ox}$ complex the spectral changes occur within the dead time (1 ms) of a stopped-flow instrument. We have extended our studies of complex assembly to selected mutant forms of TMADH altered at the position of residue Tyr-442 in native TMADH. Mutation of Tyr-442 on the surface of TMADH compromises substantially the rate of electron transfer from the 4Fe-4S center of TMADH to the FAD of ETF (18), Table I. We conjectured previously that the compromised rates of electron transfer observed with the mutant TMADH enzymes are attributed to impaired rates of conformational change/structural realignment in the complex required for subsequent electron transfer (18) and that the electron transfer event could be described by the following kinetic scheme (Reaction 2):

$$A + B \xrightarrow{k_{r}} \xrightarrow{k_{t}} (AB) \xrightarrow{k_{a}} \xrightarrow{k_{r}} (A'B') \rightarrow A' + B'$$

**REACTION 2**

In Reaction 2, $A$ is ETF$_{ox}$, $B$ is TMADH$_{sq}$, (AB) is a non-productive electron transfer complex, (AB)$_2$ is the productive electron transfer complex that forms following structural realignment of complex (AB)$_2$ and (A'B') is the product complex following electron transfer. An analytical rate equation for the observed electron transfer rate constant ($k_{obs}$) measured in stopped-flow studies can be derived for such a reaction (Equation 3 (18)),

![Fig. 4. Absorbance spectrum of ETF$_{sq}$ prior to and after incubation with TMADH. Method: ETF$_{sq}$ (20 $\mu$M) incubated with TMADH (1 $\mu$M) and flavin fluorescence (540 nm) monitored over a 48 h period to ensure the reaction was completed. The spectrum of TMADH was subtracted from the acquired spectrum to yield the spectrum of imprinted ETF$_{sq}$. Dashed line, ETF$_{sq}$ prior to incubation with TMADH; solid line, ETF$_{sq}$ after incubation and removal of the TMADH spectrum. Inset, difference spectrum (spectrum after incubation minus spectrum before incubation with TMADH). Conditions: 50 mM potassium phosphate buffer, pH 7.0; 25 °C.](https://www.jbc.org/content/jbc/284/16/8461/F3.large.jpg)

![Fig. 5. Kinetics of imprinting of ETF$_{sq}$ with mutant TMADH enzymes altered at residue position 442. Rates for reactions performed with wild-type Y442F, Y442G, and Y442L TMADH enzymes are represented by squares, triangles, circles, and inverted triangles, respectively. Imprinting reactions performed with wild-type TMADH and αR237A were substantially compromised (not shown) as a result of the known very slow rate of complex assembly determined by difference spectroscopy ETF (22). Conditions: as for Fig. 3.](https://www.jbc.org/content/jbc/284/16/8461/F4.large.jpg)
In the regime where the intrinsic electron transfer rate \( k_{eT} \) is fast, and where \( k_r \gg k_{-r} + k_{r}[A] \), Equation 3 is approximated by a linear relationship between \( k_{\text{obs}} \) and \([A]\), and this situation pertains for the wild-type complex (18). Saturation behavior is observed for mutant complexes altered at residue 442, and this was previously inferred to represent a rate-limiting conformational change (conversion of \((AB)_1\) to \((AB)_2\)), rather than a rate-limiting intrinsic electron transfer rate \( (k_{eT}) \) (18)). Consistent with this model, we provide evidence in this report for impaired structural realignment during mutant complex formation by extending our difference spectroscopy studies to complexes formed with mutant TMADH enzymes. The development of spectral change accompanying the assembly of ETFsq with the Y442F, Y442L, and Y442G mutant TMADH ox enzymes (Fig. 1) occurs with rates \( 2 \) orders of magnitude less than the wild-type complex (Table I). The development of spectral change for a mixture of ETFox with Y442G TMADH is also substantially slower than that seen for the corresponding wild-type complex. The development of the spectral changes accompanying the assembly of Y442F and Y442L mutant TMADHox enzymes with ETFox are too rapid to observe using the stopped-flow method. It is clearly not possible to perform interaction studies with one-electron-reduced TMADH and ETFox in the absence of electron transfer. Nevertheless, our difference spectroscopy studies with mutant TMADHox ETFsq complexes indicate that mutation of Tyr-442 in TMADH impairs complex assembly and that this is likely to extrapolate to complex formation with one-electron-reduced TMADH and ETFox. Also, our structural model of the electron transfer complex (25) suggests that there is a close interaction between Tyr-442 (TMADH) and \( \alpha \text{Arg}-237 \) (ETF), and that this interaction may be key to facilitating the structural reorganization required to form a productive electron transfer complex. Our recent difference spectroscopy studies with the \( \alpha \text{R}237\alpha \) mutant ETF (22), which also indicate impaired complex assembly, are consistent with this model of complex assembly.

A key observation of our work is that the spectral changes accompanying complex assembly with the mutant TMADH enzymes are generally much slower than the electron transfer rates in these complexes measured by stopped-flow methods.
as-purified were obtained for imprinting reactions performed with ETFox and the interaction surface on TMADH. Similar results presumably as a result of the impaired interaction between ETF and the complex but that one or more metastable forms of the complex formation with TMADH in facilitating the conformational change. The imprinting reaction, however, appears to be catalyzed only by TMADH. For example, the same reaction performed with human MCADH, which is a primary redox donor of human ETF, leads to a very slow (over 24 h) 3-fold increase in flavin fluorescence for M. methylotrophus ETFox. However, the FAD could not be reduced enzymatically following incubation with MCADH, indicating that the ETF is no longer redox-active. Given the prolonged incubation time (>24 h) the fluorescence increase is most likely the consequence of FAD release. Additionally, fluorescence emission increases were not observed on incubating the physiological redox partners human MCADH and human ETFox, suggesting that structural imprinting is restricted to M. methylotrophus ETF and TMADH.

**Solution Structural Properties of Imprinted ETF**—From our previous investigations of the solution structure of M. methylotrophus ETF using small-angle x-ray scattering, we were able to show that domain II of ETF is conformationally mobile with respect to domains I and III. It became clear that changes in the orientation, or restricted movements, of domain II would influence noticeably the scattering behavior of ETF for scattering values \( s > 0.02 \text{ Å}^{-1} \). Consequently, the direct comparison of the scattering signal of the imprinted ETF with that of the native protein in the above-mentioned scattering regime indicates no overall conformational difference, thus ruling out any change in domain characteristics after the imprinting reaction. However, because the spatial resolution obtainable in the present SAXS study is not better than 12.5 Å, local conformational changes such as those resulting from side-chain reorientations (e.g. in the close vicinity of the FAD moieity) cannot be excluded. The conformational change giving rise to imprinted ETF is therefore of a more subtle nature than can be detected by SAXS. This suggests that the conformational restructuring of ETF upon imprinting is small and localized near the FAD. One possible cause of the conformational change that results from the imprinting reaction comes from considering the time scale of the imprinting. This (half-life ~ 30 min) corresponds to the time scale expected for proline cis-trans isomerization. The suggestion that a proline residue may be responsible is further substantiated by analysis of the structure of the active site; there is a proline residue (ωPro-238) immediately following ωArg-237, a residue we have shown in this study to affect complex formation and in previous work to affect the redox potential of the FAD (22). Furthermore, a proline does not occur in this position in other ETFs, which do not undergo the imprinting reaction. We are currently targeting this proline by mutagenesis to study how it influences the imprinting reaction.

**Dynamic Properties of the ETF-bound FAD and Implications for Electron Transfer**—Measurement of the fluorescence anisotropy and lifetime of proteins that contain fluorescent cofactors can provide valuable information on the dynamic properties of the fluorophore. These analyses provide information about structural/dynamic properties of the protein and/or the environment of the fluorophore within the protein. The imprinting reaction of ETF is characterized principally by an increase in the fluorescence emission of the FAD cofactor; anisotropy and lifetime measurements for the FAD cofactor in both ETF and imprinted ETF, and for free FAD and free FMN, were therefore performed to gain additional information on the properties of the fluorophore. Our data reveal that the lower
anisotropy values for imprinted ETF$_{sq}$ and ETF$_{ox}$, compared with the corresponding values for ETF in the absence of TMADH, result from reduced rotation correlation times, suggesting looser binding of the fluorophore to ETF. Non-physiological, inorganic redox partners are useful tools in the study of protein-mediated electron transfer reactions, and as probes of structural change in engineered redox proteins (e.g. Ref. 18). The kinetics of electron transfer from ETF$_{sq}$ to the ferricenium ion revealed that imprinting of ETF$_{sq}$ accelerates electron transfer to the ferricenium ion. Our stopped-flow studies of electron transfer thus provide additional evidence for an altered conformational state in the imprinted form of ETF$_{sq}$ and suggest that the bound FAD is more accessible to the ferricenium ion, consistent with the looser binding inferred from our fluorescence anisotropy studies. Loose binding of the FAD may also favor electronic coupling to the physiological electron donor TMADH, because in our model of the complex we suggested the flavin isoalloxazine ring might penetrate a small groove on the surface of TMADH in the vicinity of Tyr-442 (28). In the crystal structures of human and P. denitrificans ETF the isoalloxazine ring interacts closely with residues in ETF. By analogy, the isoalloxazine ring of M. methylotrophus ETF may need to be released from similar interactions to enable closer interaction with the groove on the surface of TMADH to facilitate efficient electron transfer. Providing evidence for improved electronic coupling with TMADH as a result of imprinting ETF is a focus of our future work.

**Significance to Biological Electron Transfer Reactions in Vitro**—There are very few structures solved of soluble, physiological, and transiently formed electron transfer complexes. Examples include the methylamine dehydrogenase-amicyanin-cytochrome c (32) and cytochrome-c peroxidase-cytochrome c (33) complexes. In these cases, the structures of the protein components in the complex resemble those of the uncomplexed molecules, giving rise to a colliding “billiard ball” representation of complex assembly. These structural studies thus provide little insight into the extent and role of dynamics in electron transfer. However, kinetics studies have revealed that small-scale conformational changes occur in electron transfer from cytochrome c to ferricytochrome b$_5$, from cytochrome c to plastocyanin (35, 36), and from methanol dehydrogenase to cytochrome c$_{551}$ (37). Also, large-scale conformational change has been suggested, from x-ray studies, as a prerequisite for efficient electron transfer; for example cytochrome bc$_2$ complex (38), sulfite reductase (39), and, to a lesser extent, the components of the bovine adrenodoxin reductase-adrenodoxin eT complex (40). Our own studies have illustrated that ETF also undergoes large-scale conformational change on electron transfer complex formation (25–27), as illustrated by the “rearrangement” step in Reaction 2. The results we present here, consistent with our earlier conclusions (18), suggest that this rearrangement does not lead to the thermodynamically most stable state (which is formed too slowly) but to one or more (more rapidly formed) metastable states that are productive electron transfer complexes.

In addition to this large-scale structural rearrangement on binding, our studies also reveal that the properties of ETF can be affected by transient interaction with TMADH. This has important implications for our understanding of biological electron transfer reactions in vivo, because ETF encounters TMADH constantly in the cell. This suggests that the kinetic scheme we proposed previously (18) for the oxidative half-reaction (Reaction 2) is applicable to the in vivo situation with the modification that the ETF is in the structurally imprinted, rather than non-imprinted, form. Thus, Reaction 2 becomes Reaction 3,

\[
A^+ + B \overset{k_{a}}{\rightleftharpoons} (A'B)_1 \rightarrow (A'B') \rightarrow A' + B^-
\]

**Reaction 3**

where A’ is (imprinted) ETF$_{sq}$, B is TMADH$_{sq}$, (A’B)$_1$ is a non-productive (imprinted) electron transfer complex, (A’B)$_2$ is the (imprinted) productive electron transfer complex that forms following structural realignment of complex (A’B)$_3$, and (A’’B) is the (imprinted) product complex following electron transfer. The situation in vitro is somewhat more complex, because initially the ETF is in the non-imprinted form. However, as the reaction proceeds (some of) the ETF will become imprinted, leading to a more complex kinetic scheme (Reaction 4),

\[
A + B \overset{k_{a}}{\rightleftharpoons} (A'B)_1 \overset{k_{ET}}{\rightarrow} (A'B') \rightarrow A' + B^-
\]

**Reaction 4**

This reaction begins along the top path. However, as ETF interacts with TMADH to undergo large-scale conformational change and form the productive electron transfer complex, the ETF can undergo the imprinting reaction; once the ETF is structurally imprinted, the reaction proceeds along the bottom path, corresponding to the in vivo scheme.

**In vivo**, because of the higher concentrations of both ETF and TMADH, the two proteins are likely to exist in the complexed form for the vast majority of the time; moreover, this complex is likely to have progressed from an initial metastable form to the thermodynamically most stable form before the electron transfer event takes place. In turn this suggests that one or more metastable forms may not be directly relevant to the reaction in the cell. To understand more clearly the conditions in vivo, our efforts are now focused on elucidating in detail the electron transfer reactions of imprinted ETF with TMADH. Thus, our present studies suggest that care should be exercised when interpreting the results of in vitro studies, particularly when equating them to physiological reactions.

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