The interaction between the physiological electron transfer partners trimethylamine dehydrogenase (TMADH) and electron-transferring flavoprotein (ETF) from *Methylophilus methylotrophus* has been examined with particular regard to the proposal that the former protein “imprints” a conformational change on the latter. The results indicate that the absorbance change previously attributed to changes in the environment of the FAD of ETF upon binding to TMADH is instead caused by electron transfer from partially reduced, as-isolated TMADH to ETF. Prior treatment of the as-isolated enzyme with the oxidant ferrocyanide essentially abolishes the observed spectral change. Further, when the semiquinone form of ETF is used instead of the oxidized form, the mirror image of the spectral change seen with as-isolated TMADH and oxidized ETF is observed. This is attributable to a small amount of electron transfer in the reverse of the physiological direction. Kinetic determination of the dissociation constant and limiting rate constant for electron transfer within the complex of (reduced) TMADH with (oxidized) ETF is reconfirmed and discussed in the context of a recently proposed model for the interaction between the two proteins that involves “structural imprinting” of ETF.

Trimethylamine dehydrogenase (TMADH, EC 1.5.99.7) isolated from the bacterium *Methylophilus methylotrophus* (isolate W2A1) is an iron-sulfur-containing flavoprotein consisting of two identical, catalytically independent subunits. Each subunit has a molecular mass of 83 kDa and contains an unusual covalently linked 6-S-cysteinyl FMN cofactor (linking the flavin via its 6-position to Cys30 of the polypeptide), a bacterial ferredoxin-type [4Fe-4S] center, and one equivalent of tightly bound ADP having an unknown function (1–7). The enzyme catalyzes the oxidative demethylation of trimethylamine (TMA) to dimethylamine and formaldehyde (presumably through an imine intermediate that spontaneously hydrolyzes once dissociated from the enzyme). This reductive half-reaction of the catalytic cycle results in two-electron reduction of the enzyme shown in Reaction 1.

\[ E_{ox} + (CH_3)_3N + H_2O \rightarrow E_{red} + (CH_3)_2NH + HCHO + 2H^+ \]  

**REACTION 1**

The catalytic cycle is completed by an oxidative half-reaction that involves removal of reducing equivalents from the enzyme via its [4Fe-4S] center (after electron transfer from the FMN site) by electron-transferring flavoprotein (ETF), the natural electron acceptor for TMADH (1). ETF is a 63-kDa αβ heterodimer containing one equivalent of FAD and one equivalent of AMP (like the ADP of TMADH, of unknown function) (8), and operates in a one-electron oxidation-reduction cycle between the oxidized FAD and the anionic semiquinone FAD\(^{-}\) oxidation states (9). However, recently it has been shown that ETF can be readily reduced to the hydroquinone form by both trimethylamine and sodium dithionite reduction when it is bound to TMADH (10). The ETF semiquinone passes its reducing equivalent to an ETF:CoQ oxidoreductase and thus serves as an electron carrier between various catabolic enzymes and the principal respiratory chains of a variety of organisms (including man) (11, 12).

TMADH is an interesting example of a complex flavoprotein that possesses multiple redox-active centers and is a convenient system in which to study biological electron transfer. TMADH can take up a total of three electrons per subunit upon reduction: two at the FMNH\(_2\) and one at the iron-sulfur center. The enzyme with two electrons per subunit (TMADH\(_{2e}\)), the reduced form generated on reduction by trimethylamine, is not fully reduced and has two possible distributions: fully reduced FMNH\(_2\) with oxidized [4Fe-4S] or flavin semiquinone and reduced [4Fe-4S]. The equilibrium between these two distributions is governed by the reduction potentials of the centers and is pH-dependent. At pH 7.0 the former distribution is favored by a factor of approximately two (13).

Previous studies have examined both intramolecular electron transfer within TMADH (13) and intermolecular electron transfer between TMADH and ETF (10, 14–17). The former work has demonstrated that electron transfer from FMN to the iron-sulfur center in TMADH occurs rapidly, with \(k_{ET} > 1000\) s\(^{-1}\), and is not rate-limiting to overall catalysis. Electron transfer on to ETF has been studied by following the reaction of reduced TMADH with oxidized ETF, varying the concentration of the latter (14, 15). These two studies have shown that the observed rate constant for electron transfer exhibits hyperbolic dependence on [ETF], from which an apparent limiting \(k_{ET} \approx 180\) s\(^{-1}\) and a dissociation constant for binding of TMADH\(_{red}\) and ETF\(_{ox}\) of 10 μM have been determined. Assuming a decay parameter \(β\) for electron transfer as a function of distance of 1.4 Å\(^{-1}\), this value for the limiting rate of electron transfer is consistent with...
Electron Transfer between TMADH and ETF

a donor-acceptor distance of ~12 Å; approximately the extent to which the [4Fe-4S] is buried in TMADH. Tyr442 lies at the surface of TMADH, constitutes one side of a shallow cleft on the surface of the protein nearest the buried [4Fe-4S] center (7), and it is likely that ETF docks close to Tyr442 in forming the protein-protein complex. Kinetic studies of the TMADH mutants Y442F, Y442L, and Y442G have shown that mutations at this site significantly influence electron transfer from TMADH to ETF; at 25°C, the Y442G mutant exhibits a ~30-fold decrease in the limiting rate constant for electron transfer and a 10-fold increase in \( K_d \) (15).

Recently, the reaction of reduced TMADH with ETF has been reexamined (17), not only with wild-type enzyme but also several site-directed mutants at Tyr442, which had previously been investigated (15). Whereas results with the mutant TMADH forms were largely consistent with the earlier study, the wild-type enzyme was found to exhibit kinetics with a linear dependence on [ETF] rather than the hyperbolic dependence found in the two earlier studies. Based on this observation, a model was proposed in which mutations at Tyr442 had very large effects on the rate of electron transfer from the iron-sulfur center of TMADH to the FAD of ETF, although the effect was masked by a rate-limiting conformational change with the wild-type enzyme. In subsequent studies (18), it was suggested that subsequent to this rate-limiting conformational change, ETF was converted to a form that was far more kinetically competent to be oxidized or reduced. This conformational change was proposed to persist for some time even after dissociation of ETF from TMADH, and the overall process was termed "structural imprinting" (18).

In an effort to resolve these conflicting interpretations in the literature and to clarify the kinetic behavior of the oxidative half-reaction of TMADH with ETF, we have further examined the spectroscopic changes and kinetics of their interaction. In particular, the protein concentration and oxidation state dependence of spectral changes associated with the formation of complex of TMADH and ETF have been reexamined. The results indicate that the spectral changes previously attributed to complex formation between TMADH and either ETFox (10) or ETFsq (19) are instead attributed to electron transfer processes, which can be partially reversed under certain conditions. In addition to equilibrium studies of formation of the TMADH-ETF complex, kinetic experiments with wild-type and a Y442G mutant have also been reexamined. The results are fully consistent with the two earlier studies, and we find no evidence to support a model for electron transfer between TMADH and ETF that involves a rate-limiting conformational change within the TMADH-ETF complex or, by corollary, a profound effect on electron transfer upon mutating Tyr442. The results are also inconsistent with the formation of an imprinted state of ETF upon prior incubation with TMADH.

**EXPERIMENTAL PROCEDURES**

Chemicals—Mono- and di-potassium phosphate and TMA were obtained from Sigma. Sodium dithionite was from Virginia Chemicals. Ferricenium hexafluorophosphate was prepared as described by Lehman et al. (20, 21). TOPO-TA (with TOP 10 electrocompeotent cells) and pTrRecHis2A were purchased from Invitrogen. All enzymes used in the molecular biology experiments (including NcoI and XhoI) were purchased from MBF Fermentas, except for Pfu DNA polymerase, which was purchased from Stratagene. All bacterial media were purchased from Fisher.

**TMADH and ETF Purification**—M. methylotrophus \( W_A \) was grown on TMA as sole carbon source, and TMADH was purified as described by Steenkamp and Gallup (9). Again, Sephadex G-200 in the gel filtration step of the purification was replaced with Sephacryl S-200. Because ETF was isolated typically partially reduced, it was oxidized by reaction with ferricenium hexafluorophosphate prior to use (yielding ETFox). Residual oxidant was removed by passage through a Sephadex G-25 column equilibrated with 50 mM phosphate buffer, pH 7.0. The concentration of ETF was determined from the absorbance at 438 nm using an extinction coefficient of 11.3 mM \(^{-1} \text{cm}^{-1} \) (9).

**Purification of Recombinant Trimethylamine Dehydrogenase**—The gene encoding the previously reported TMADH Y442G mutant (15) was obtained by a modified version of the QuikChange mutagenesis protocol (Stratagene) using the wild-type gene cloned into the TOPO-TA maintenance vector (to be described elsewhere) and the mutagenic nucleotide: 5′-GGTGCAGTGAGCGGTACCCCGG-3′. The mutant gene was subcloned into the pTrRecHis2A vector and the mutation confirmed by sequencing. This vector was used to transfect electrocompeotent Escherichia coli BL21 DE3 cells and overexpress the recombinant protein. For the recombinant wild-type and mutant enzymes, a rapid purification protocol was adapted and modified from the previously described method for the purification of trimethylamine dehydrogenase from bacterial \( W_A \) (1). Transfected E. coli BL21 DE3 cells from above were grown in 2 liters of TPP medium containing 0.1 g/liter flavin mononucleotide (FMN), 0.1 g/liter riboflavin, 0.2 g/liter ferrous ammonium sulfate, and 100 mg/ml ampicillin. Cells were grown at an \( A_{600} \) of 0.6, induced with 400 \( \mu \)M isopropyl-\( \beta \)-thiogalactoside, grown for 24 h at 28°C, and harvested by centrifugation (7000 \( \times g \) for 10 min). Cells were resuspended in 50 ml of 50 mM potassium phosphate buffer, pH 7. Crude lysate from cell lysis/sonication was loaded onto a DEAE column and eluted using a linear 0–0.5 M NaCl gradient. Fractions containing recombinant TMADH were collected and pooled based on purity (UV-visible spectrum, SDS-PAGE), and then concentrated. The enzyme was then loaded onto an S-200 column pre-equilibrated with potassium phosphate buffer and eluted with the same buffer. With this improved construct, increased levels of FMN incorporation were evident, typically reaching 70%. Enzyme concentrations were determined spectrophotometrically using an extinction coefficient of 27,300M \(^{-1} \text{cm}^{-1} \) at 443 nm, after correction for the flavin enzyme (6).

**Binding Studies of TMADH and ETF**—Solutions of TMADH were placed in a tonometer and made anaerobic by alternately evacuating and flushing with O2-free argon. Using a Hamilton syringe, 1.2-ml samples of TMADH at the desired concentration were placed in one side of a split-cell cuvette that was sealed with a rubber septum and made anaerobic by flushing with O2-free argon. A solution of oxidized ETF (ETFox) of the same concentration was made anaerobic in the same way, and 1.2 ml of this solution was then placed in the other side of the cuvette. The visible spectra of TMADHox and ETFox of 10 \( \mu \)M (14), a 10-fold greater concentration of the two proteins in the present experiment should ensure essentially complete formation of the protein complex (assuming the \( K_d \) is not greatly affected by the oxidation state of TMADH). Because concentrations used in the experiments were limited by the light path of the cuvette and the non-linearity of Beer’s law at high optical density, the concentrations used here could be no higher than 100 \( \mu \)M. The identical protocol was used to obtain the difference spectrum associated with formation of the TMADH and ETFox complex. ETFsq was obtained by titration of ETF with sodium dithionite.

**Simulation of Spectral Changes Associated with Electron Transfer from TMADH to ETF**—The readily obtained spectra of TMADHox, TMADHsq, ETFox, and ETFsq were used to calculate the difference spectrum of TMADHsq – ETFox and the difference spectrum of ETFsq – ETFox. These two difference spectra were then used to obtain a simulation of the spectral change associated with electron transfer from TMADH to ETF.

**Treatment of TMADH with Phenylhydrazine Hydrochloride**—Phenylhydrazine-inactivated TMADH (TMADHinact) was prepared according to the method described previously (23): 50 \( \mu \)M TMADH in

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0.1 M potassium phosphate buffer, pH 7.7, was incubated with 2 mM phenylhydrazine at 30 °C for 4 h. The solution was then filtered, concentrated and passed through a Sephadex G-25 column to remove excess phenylhydrazine. The concentration of TMADH thus obtained was determined using an extinction coefficient of 21.4 mm⁻¹ cm⁻¹ at 410 nm (6).

Kinetics of Electron Transfer between TMADH and ETF—Kinetic experiments were carried out using a Kinetic Instrument Inc. stopped-flow apparatus equipped with an On Line Instruments Systems (OLIS) model 3920Z data collection system, or an Applied Photophysics SX 17 MV stopped-flow spectrophotometer. Anaerobic native TMADH, TMADHinact, or mutant TMADH Y442G was made anaerobic in a glass cuvette by alternate cycles of evacuation and flushing with argon, and reduced by titration with sodium dithionite delivered via a titration syringe. Spectral changes were monitored via a side-arm cuvette attached to the tonometer. Fully oxidized ETF was prepared as described above and made anaerobic as above in a tonometer. The concentration of reagent in excess in the stopped-flow experiment (either ETF or TMADH) was at least five times greater than that of the limiting reagent to ensure pseudo first-order conditions. Kinetic transients obtained after mixing reduced TMADH with ETF were fitted to sums of exponentials using software provided by the manufacturers (24). Although well-established anaerobic procedures were used throughout (25, 26), it is to be noted that the reduced forms of both TMADH and ETF are unusually unreactive toward oxygen (each requiring hours in air to become reoxidized). In addition, no evidence of oxygen contamination (e.g., a reduced absorbance changes seen in the course of an experiment) was observed in any of the studies reported here.

**RESULTS**

The Binding of As-isolated TMADH to Oxidized ETF and ETF Semiquinone—The spectral change observed upon mixing TMADHai and ETFox, is shown in Fig. 1 A (solid line) and is in good agreement with that reported previously (10). Previous studies of a variety of flavoproteins (27–31) have reported spectral changes similar to that seen here on mixing TMADHai with ETFox. Examples include the binding of aromatic amino acids or benzoate to ε-amino acid oxidase (27, 28), p-methoxybenzaldehyde to old yellow enzyme (29), tartrone to lactate oxidase (30), and 8-bromoxanthine to oxidized xanthine oxidase (31). These spectral changes have been interpreted to be arising from moving a normal non-covalently modified flavin from a more hydrophilic environment to a more hydrophobic one. Based on this earlier work, we have previously proposed (10) that the spectral change shown in Fig. 1 A (solid lines) is caused by a perturbation of the environment of FAD in ETF (rather than the spectrally distinct 6-S-cysteinyl FMN of TMADH) upon formation of the TMADH-ETF complex. If this is true, then it should be possible to use this spectral change to obtain a dissociation constant for ETF binding to (oxidized) TMADH. Fig. 1B shows the dependence of the observed spectral change on protein concentration, with results that are at least qualitatively consistent with a $K_d$ of $10 \mu M$. The observed spectral change, however, also turns out to be very similar to the difference spectrum obtained by Huang et al. (14) for the oxidation of TMADHinact or of reduced phenylhydrazine-inactivated TMADH by ETFox. This observation raises the question of whether the observed spectral change in the present experiment arises from complex formation between TMADH and ETF or from electron transfer processes.

In addition to the spectral change attributed to binding of TMADH to oxidized ETF, a very large spectral change associated with formation of a complex between as-isolated TMADH and ETF semiquinone (ETFsq), with an extinction change of over 16.0 mm⁻¹ cm⁻¹ at 460 nm, has been reported (18, 19). This extinction change exceeds the extinction coefficient for the anionic semiquinone at this wavelength, and the reported spectral change in fact closely resembles that seen upon reduction of TMADH. We have further examined the spectral change associated with mixing TMADHsq with ETFsq (reduced by titration with sodium dithionite), rather than the fully oxidized ETFox. The observed spectral change is shown in Fig. 1A (dotted line). Surprisingly, this change is not at all like that reported previously (19) but rather appears to be the mirror image of that seen in the experiment using TMADH and ETFox. It seems very likely that in the earlier study using ETFsq the observed spectral change seen upon mixing TMADHsq with ETFsq was caused by overtitration of ETF with excess reductant which reduced TMADH when the two protein solutions were mixed.

The observed mirror image relationship of the spectral changes seen for the (TMADHai + ETFox) experiment, on the one hand, and the (TMADHsq + ETFsq) experiment on the other, is extremely surprising if, as has been previously concluded, the former is in fact caused by a change in the environment of the FAD of oxidized ETF because the spectral signa-
 Electron Transfer between TMADH and ETF

Fig. 2. Spectral changes associated with the reaction of the TMADH-ETFox and TMADHinact-ETFox protein complex. TMADHox was obtained by pretreatment with ferricenium hexafluorophosphate. The spectral change is obtained by the spectrum for after-mixture minus that for before-mixture. A, spectral changes for 15 μM TMADH-ETFox and TMADHox-ETFox protein complex. B, spectral changes for 30 μM TMADH-ETFox and TMADHox-ETFox protein complex.

The question is thus raised as to whether the observed spectral changes might instead reflect electron transfer processes (from TMADH to ETF in the first experiment, and partial reversal, from ETFox to TMADH, in the second) rather than complex formation.

The spectral change is obtained by the spectrum for after-mixture minus that for before-mixture. A, spectral changes for 15 μM TMADH-ETFox and TMADHox-ETFox protein complex. B, spectral changes for 30 μM TMADH-ETFox and TMADHox-ETFox protein complex.

The difference spectrum for electron transfer between TMADH and ETF represents the sum of the spectra of the final states of enzymes (TMADHox + ETFred) minus those for the initial states (TMADHred + ETFox), each species having a fully defined visible absorption spectrum. As a result, the expected spectral change for (TMADHox − TMADHred) + (ETFred − ETFox) can be calculated directly. This ΔA is shown in Fig. 1C, where it can be seen that the calculated spectral indeed provides a very good fit to the experimentally observed spectral change. This result supports the conclusion that the experimentally observed spectral change is a manifestation of interprotein electron transfer and not of complex formation, per se.

Kinetics of the Reaction of Reduced TMADH with ETFox—On the basis of the above, it is not possible to determine a Kd for the TMADH-ETF complex from absorbance changes seen with binding TMADHox and ETFox as a function of the concentrations of the proteins. The only reliable estimates for the Kd for ETF binding to TMADH remain those determined kinetically from the [ETF] dependence of the rate constant for the reoxidation of TMADHred (14, 15) and from ultracentrifugation experiments (32, 33), which yield values for Kd of 5–10 μM. Although the concentration dependence of the reaction of reduced TMADH and ETF has been reported (14) and confirmed (15) to be hyperbolic in nature, recently a linear dependence for the wild-type enzyme has been reported (18). In order to further characterize the interaction of TMADH with ETF in light of the above, the kinetics of the reaction with ETF with two different reduced forms of TMADH (native TMADH and enzyme that has been inactivated by treatment with phenylhydrazine to render the flavin redox-inert, Ref. 6) have been reexamined. Previous studies have used excess ETF to react with limiting TMADHred. Although a wider range of concentration for ETF would be desirable, the strong absorbance of both TMADH and ETF and the need to work in an absorbance range where Beer’s law applies significantly limits the experimentally accessible concentration range. As a result, we have performed experiments reversing the concentrations of the two proteins, using a pseudo first-order excess of TMADH rather than ETF. In both cases, the reaction can be followed conveniently at 370 nm, where the accumulation of the anionic semiquinone of ETF can be monitored.

Native or phenylhydrazine-inactivated TMADH was reduced by titration with sodium dithionite, following the course of the reduction spectrophotometrically. Fig. 3A shows a typical kinetic transient seen for the reaction of reduced TMADHinact (5 μM) with ETFox (50 μM). Reduced TMADHinact has only one reducing equivalent in the iron-sulfur center, and because it can react with only a single equivalent of ETF, the overall reaction is greatly simplified. As seen previously (14), the observed transients are best fit as the sum of two exponentials, with kfast = 128 s⁻¹ and kslow = 15.3 s⁻¹ (accounting for 57 and 43% of the total observed spectral change, respectively); kfast for this and three other concentrations of ETF (under pseudo first-order conditions) are plotted in Fig. 3D (filled squares). Fig. 3B shows a transient for the reaction when the concentrations of TMADH and ETF are reversed, i.e. 50 μM reduced TMADHinact and 5 μM ETFox. The transients are again biphasic, with the fitting results giving kfast = 140.3 s⁻¹ and kslow = 45.3 s⁻¹ (accounting for 61 and 39% of the observed ΔA, respectively), in reasonable agreement with the experiment shown in Fig. 3A. Fig. 3C shows the transient observed for the reaction of 80 μM TMADHinact with 7 μM ETF, yielding a kfast of 171 s⁻¹;
The data over the total concentration range that is experimentally accessible again show hyperbolic character for wild-type enzyme, not a linear concentration dependence with an intercept at the origin (19), with a $K_d$ of 10 $\mu$M, and a limiting rate constant for electron transfer of 170 s$^{-1}$ (Fig. 3D, solid line). The new data are not only quantitatively in agreement with the previous studies, but also specifically corroborate the asymptotic nature of the plot in the high concentration regime. Although the curvature in the combined data sets shown in Fig. 3D is readily evident, a linear fit to the data could be achieved if a non-zero y-axis intercept is allowed. Such a case would correspond to a simple one-step reversible process, with an association rate constant obtained from the slope of the plot and a dissociation rate constant from the y-axis intercept, with no formation of a discrete TMADH-ETF complex. In addition to the inferior linear fit to the data, we note that complex formation between TMADH and ETF is observed crystallographically as well as by ultracentrifugation studies (see below). We therefore do not favor the interpretation that the reaction proceeds in a single, reversible step.

To compare with native TMADH and re-check the role of Tyr$^{442}$ in the interaction of TMADH and ETF, we reexamined the concentration dependence of the mutant Y442G with ETF. As shown in Fig. 4, the mutant reacts more slowly than wild-type enzyme with ETF but again exhibits a hyperbolic dependence on [ETF]. Compared with wild-type enzyme, $k_{obs}$ is reduced ~40-fold to 4.0 s$^{-1}$ and $K_d$ increased 8-fold to 80 $\mu$M, consistent with both previous studies (15, 18). It is evident that the mutation has a significant effect on the electron transfer kinetics, but the hyperbolic concentration dependence seen with wild-type enzyme is retained.

**Further Assessment of the Imprinting Phenomenon**—Based on both kinetic and fluorescence studies, it has recently been proposed that a rate-limiting conformational change in ETF within the TMADH-ETF complex occurs (18), and that ETF is subsequently converted to a structurally imprinted form. By this it is meant that binding induces a conformational change in ETF that makes it kinetically more easily reduced and which persists for a period of time even after dissociation from TMADH (18). TMADH is specifically proposed to catalyze the conversion of unimprinted to imprinted ETF. It has been suggested that such imprinted ETF differs structurally from assimilated ETF in only the immediate vicinity of FAD because overall molecular conformations did not show much difference by small angle x-ray scattering studies (25). That a conformational change of some sort must occur for formation of a TMADH-ETF complex is readily evident, because from their crystal structures the surfaces of the two proteins that undoubtedly must interact are both distinctly concave and do not complement one another. On the other hand, the properties

![Image](http://www.inlift.org/)

Fig. 3. Time course observed for the reaction of reduced phenylhydrzone-inactivated TMADH with oxidized ETF. Absorbance changes observed at 370 nm after mixing in a stopped-flow apparatus are plotted versus time. The reaction conditions are 50 mM potassium phosphate, pH 7.1, 25 °C. The smooth line represents fits of the data to exponentials of the form $\Delta A(t) = \Delta A_0 \exp(-k_t t)$ where $\Delta A$ and $k_t$ represent the absorbance change and observed rate constant exhibited by the nth kinetic phase, respectively. A, reaction of dithionite reduced phenylhydrzone-inactivated TMADH with ETF$_{ox}$. The concentration before mixing are: [ETF$_{ox}$] = 50 $\mu$M, [TMADH$_\text{inact}$] = 5 $\mu$M. Data are fitted to the sum of two exponentials: $k_{fast} = 128$ s$^{-1}$ and $k_{slow} = 15.3$ s$^{-1}$. B, same as A except the concentrations before mixing are: [TMADH$_\text{inact}$] = 50 $\mu$M, [ETF$_{ox}$] = 4.4 $\mu$M. Data are fitted to the sum of two exponentials: $k_{fast} = 140.3$ s$^{-1}$ and $k_{slow} = 45.3$ s$^{-1}$. C, same as A, except the concentrations [TMADH$_\text{inact}$] = 160 $\mu$M, [ETF$_{ox}$] = 15 $\mu$M. Data were fitted at the first 40 ms: $k_{obs}$ is reduced ~40-fold to 4.0 s$^{-1}$ and $K_d$ increased 8-fold to 80 $\mu$M, consistent with both previous studies (15, 18). It is evident that the mutation has a significant effect on the electron transfer kinetics, but the hyperbolic concentration dependence seen with wild-type enzyme is retained.

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attributed to the imprinted ETF itself are unusual. It is difficult to imagine, for example, how ETF maintains a metastable conformation even after dissociating from TMADH, particularly in light of the apparently highly dynamic nature of the ETF structure in solution (see below).

In order to critically evaluate whether TMADH catalyzes the conversion ETF to a structurally imprinted form, we have compared the kinetics of the reaction of excess native TMADH$_{w}$ with ETF$_{ox}$, in the absence and presence of stoichiometric concentrations of TMADH$_{ai}$ in the ETF solution. If imprinting occurs, ETF in the latter experiment should be converted to its imprinted form, and significantly faster kinetics should be observed. The results of such an experiment (see Supplementary Materials) yield $k_{fast} = 128.7$ s$^{-1}$ and $k_{slow} = 1.3$ s$^{-1}$ without TMADH$_{ai}$ in the ETF solution and $k_{fast} = 140.3$ s$^{-1}$ and $k_{slow} = 0.79$ s$^{-1}$ in its presence. We note that the observed rate constants at the concentration of ETF used is consistent with the data shown in Fig. 3D. Although the second reaction is some 9% faster than the first, it is evident that the addition of TMADH$_{ai}$ to the ETF prior to reaction does not have the profound effect expected were ETF to have become imprinted. We conclude that TMADH does not catalyze the conversion of ETF to a new imprinted form that has significantly faster electron transfer kinetics.

DISCUSSION

The essentially mirror image relationship of the spectral changes seen on mixing TMADH$_{w}$ with ETF$_{ox}$ on the one hand, and TMADH$_{ai}$ with ETF$_{ox}$ on the other, suggests strongly that these changes do not arise from a change in the environment of the (oxidized) FAD of ETF. Consistent with the interpretation that the observed spectral change is instead caused by interprotein electron transfer, the absorbance change is essentially abolished on pretreatment of as-isolated TMADH with the oxidant ferricenium prior to reaction with ETF$_{ox}$. That the inverse spectral change is seen when TMADH$_{ai}$ is mixed with ETF$_{ai}$ indicates that electron transfer in the reverse of the physiological direction occurs (although to only a small degree) given the magnitude of the spectral change. In the TMADH$_{ai}$/ETF$_{ox}$ experiment (Fig. 1D). A comparison with the previous results of Huang et al. (14) indicates that the observed spectral change is indeed very similar to that expected for electron transfer, and only coincidentally resembles that associated with changes in the flavin environment.

In both the present work and in two previous studies of the oxidative half-reaction of TMADH, we observed saturating behavior in the reaction of TMADH$_{ox}$ with ETF, with a hyperbolic dependence of the observed rate constant for electron transfer on the ETF concentration that yields a $K_d$ of ~10 µM. A comparable value has been determined in ultracentrifugation studies, with a $K_d$ of 5 µM obtained (32, 33). As shown in Fig. 3D, the present kinetic results, using TMADH in excess as well as ETF, are fully consistent with our two previous studies (14, 15).

The observation of a hyperbolic dependence of $k_{ET}$ on [ETF] with wild-type enzyme as confirmed here contrasts with the results of others (17) who have reported a linear dependence on [ETF] for wild-type TMADH (but saturating behavior for each of several mutants of TMADH). The apparent conversion of the electron transfer reaction from one of linear dependence of $k_{obs}$ on [ETF] into one having hyperbolic dependence has been incorporated into a model for the kinetic behavior of the system in which a conformational change within the TMADH-ETF complex is rate-limiting for electron transfer with wild-type enzyme, which is in turn assumed to be intrinsically very fast (17). The switch from linear to hyperbolic dependence on [ETF] going from wild-type to the mutants has been attributed to a very large effect on the intrinsic electron transfer rate $k_{ET}$ at Tyr$^{442}$. Mutations at Tyr$^{442}$ make the electron transfer process itself (rather than the proposed conformational change) increasingly rate-limiting as the side chain at position 442 is made progressively smaller. However, a model assuming an exceptionally high rate for electron transfer from wild-type enzyme, over a distance of at least 12 Å (reflecting the extent to which the [4Fe-4S] cluster is buried beneath the surface of TMADH) is not consistent with current views concerning biological electron transfer, where the polypeptide is assumed to represent a more or less uniform medium in which electron transfer occurs, with no single particularly effective pathway in operation (36) and (by corollary) no uniquely important amino acid residue. The effect of the Y442G mutation on electron transfer is certainly significant, with $k_{obs} \sim 4.0$ s$^{-1}$ and $K_d = 80$ µM determined here for the mutant (Fig. 4) compared with the wild-type enzyme values of 172 s$^{-1}$ and 10 µM, respectively (15). The effect is not nearly so profound, however, as the orders of magnitude implied in the context of a model involving a rate-limiting conformational change within the TMADH-ETF complex, subsequent to which ETF becomes imprinted (17).

As indicated above, it is evident from an examination of the crystal structures of TMADH (37) and human ETF (which is
highly homologous to the bacterial enzyme) (38) that the isolated proteins are not sterically compatible; both have surfaces that are concave in the vicinity of their respective redox-active centers (the [4Fe-4S] for TMADH and FAD for ETF). As a result, a conformational change in one or the other protein is required for the two redox-active centers to get close enough (<15 Å, Ref. 36) for effective electron transfer. Such a conformational change had in fact been previously suggested by Jang et al. (10) to account for the observation that M. methylphilicus ETF can be fully reduced to the FAD hydroquinone form both enzymatically and chemically in the TAMDH-ETF protein complex. The idea of a conformational change in ETF in the course of electron transfer is thus not controversial. The present work calls into question, however, the suggestion that any such conformational change is rate-limiting for electron transfer from TMADH to ETF, and that subsequent to such a conformational change, ETF can be converted to a structurally imprinted state in which electron transfer is particularly facile. Much of the evidence in support of structural imprinting is based on spectral changes such as those examined here. Given our demonstration here that these reflect bulk electron transfer events rather than complex formation between TMADHox and ETFimpr per se, the data in support of structural imprinting must be reconsidered. Further, the very similar kinetic behavior seen in the experiments involving reaction of excess TMADHox with ETFox with or without TMADHox present in the ETF solution (see Supplementary Materials) demonstrate that the system does not behave as expected had ETF become imprinted. Specifically, were binding of TMADH to ETF to cause ETF to convert to a structurally imprinted form, much faster kinetics should have been observed in the experiment with TMADHox present in the ETF solution than in its absence.

It is to be noted that the notion of structural imprinting as proposed for the TMADH/ETF system is also problematic from a thermodynamic standpoint. As formulated (18), the idea again is that as-isolated ETF exists in a conformation that is poorly disposed for electron transfer, but after transiently interacting with TMADH is converted to a form that exhibits more facile electron transfer properties. This form is proposed to persist for a significant period of time after ETF has dissociated from TMADH, prior to returning to the initial (and presumably more thermodynamically stable) unimprinted form. In order to comply with the First Law of Thermodynamics, this can only occur if the ETF, once converted to an imprinted state and dissociated from TMADH, only slowly reverts to the otherwise thermodynamically preferred unimprinted state in free solution. A thermodynamic box can be constructed linking the interconversion of an imprinted and unimprinted state for ETF and the binding of each to TMADH, as shown in Fig. 5. Assuming that at equilibrium the imprinted state in as-isolated ETF is present below the levels of detection (say, 1%, reflecting a hypothetical equilibrium constant of 0.01 for formation of the imprinted state) and the same is true for the unimprinted state after exposure to TMADH (with a hypothetical equilibrium constant of 100), then TMADH must have a substantially greater affinity (10^4 in the present argument) for imprinted relative to unimprinted ETF. For the system to function as proposed, the rate constant for dissociation of the imprinted ETF from TMADH must be small, but also significantly larger than that for the thermodynamically favorable conversion of the imprinted ETF to the unimprinted ETF in free solution (otherwise, a catalytic amount of TMADH would be unable to quantitatively convert a solution of ETF from the unimprinted to the imprinted form, and/or the unimprinted state would never have formed in the first place). According to this argument, relaxation to the unimprinted state in free solution must also be slow.

Recently, the crystal structure of the TMADH-ETF complex has been reported (40), providing direct confirmation of its occurrence. Interestingly, electron density for the FAD domain of ETF is unresolved, indicating that it is disordered in the co-crystal, and very likely highly dynamic. We observe the same type of disorder in the FAD domain of ETF in the TMADH-ETF complex in our own co-crystals. It is likely that this dynamic behavior allows ETF to accommodate complex formation with TMADH. A highly dynamic conformational flexibility in ETF is difficult to reconcile with the necessarily sluggish conformational changes required in the context of a structural imprinting model above, given the thermodynamic and kinetic constraints on the system. Similarly, it is unlikely that such highly dynamic conformational changes could be rate-limiting for electron transfer between TMADH and ETF. On the basis of this argument, and in conjunction with the kinetic work described here, we conclude that there is no imprinted form of ETF.

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2 The thermodynamic box presented is fundamentally equivalent to the part of Reaction 4, presented in Ref. 18, relating to the formation of the imprinted state of ETF. Here, the spontaneous conversion of unimprinted ETF to imprinted ETF in free solution is considered explicitly, however, as must formally be the case (even if the process is thermodynamically unfavorable). Also, in the present scheme the separate steps for formation of the TMADH-ETF complex and any subsequent conformational change to an electron transfer competent state are combined into a single macroscopic step, in light of the present results. It is to be emphasized that the equilibrium constant for this macroscopic step is simply the product of the equilibrium constants for the two individual steps, if they were to be considered discretely, and the argument presented remains valid.

3 W. W. Shi, C. Bell, and R. Hille, unpublished data.
