The kinetics of electron transfer between trimethylamine dehydrogenase (TMADH) and its physiological acceptor, electron transferring flavoprotein (ETF), has been studied by static and stopped-flow absorbance measurements. The results demonstrate that reducing equivalents are transferred from TMADH to ETF solely through the 4Fe/4S center of the former. The intrinsic limiting rate constant (k_{eq}) and dissociation constant (K_d) for electron transfer from the reduced 4Fe/4S center of TMADH to ETF are about 172 s^{-1} and 10 \mu M, respectively. The reoxidation of fully reduced TMADH with an excess of ETF is markedly biphasic, indicating that partial oxidation of the iron-sulfur center in 1-electron reduced enzyme significantly reduces the rate of electron transfer out of the enzyme in these forms. The interaction of the two unpaired electron spins of flavin semiquinone and reduced 4Fe/4S center in 2-electron reduced TMADH, on the other hand, does not significantly slow down the electron transfer from the 4Fe/4S center to ETF. From a comparison of the limiting rate constants for the oxidative and reductive half-reactions, we conclude that electron transfer from TMADH to ETF is not rate-limiting during steady-state turnover. The overall kinetics of the oxidative half-reaction are not significantly affected by high salt concentrations, indicating that electrostatic forces are not involved in the formation and decay of reduced TMADH-oxidized ETF complex.

Trimethylamine dehydrogenase (TMADH; EC 1.5.99.7) isolated from the methylotrophic bacterium W_3A_1 is a homodimer of molecular weight of 166,000, with each subunit containing a covalently bound 6-cysteinyl FMN coenzyme and a 4Fe/4S (ferredoxin-type) iron-sulfur center (1–4). TMADH also possesses 1 equivalent of tightly bound ADP/monomer, although the function of this cofactor remains unknown (5). The enzyme catalyzes the oxidative demethylation of trimethylamine to dimethylamine and formaldehyde, passing the pair of reducing equivalents thus obtained individually to its physiological oxidant, an electron-transferring flavoprotein (ETF), which becomes reduced to the level of the (anionic) semiquinone (4, 6–8). The ETF from W_3A_1 has been shown to be an \alphaβ dimer with molecular weight of 77,000 and contains 1 mol of FAD and AMP/mol of protein; the role of AMP is unclear.

Complete reduction of TMADH requires 3 electrons/subunit, 2 for full reduction of the FMN, and a 3rd for reduction of the iron-sulfur center. When TMADH is reduced to the level of 2 electrons/subunit, there are two possible distributions of reducing equivalents: 1) fully reduced FMN with oxidized iron-sulfur and 2) flavin semiquinone reduced iron-sulfur center. At pH 7.0, the former distribution is favored by a factor of approximately 2:1. Furthermore, although some TMADH\_2eq possesses flavin semiquinone and reduced iron-sulfur center at pH 7.0, the magnetic moments of the unpaired spins do not interact as is the case when TMADH is reduced by excess substrate, or by sodium dithionite at high pH or in the presence of the inhibitor tetramethylammonium chloride (6, 9–11). This spin-interacting form exhibits a unique EPR signal that includes half-field features and is not simply the sum of the signals for flavin semiquinone and reduced iron-sulfur center; we designate this spin-interacting form as TMADH\_2eq*.

Previous freeze-quench studies have demonstrated that when TMADH\_2eq* is mixed with ETF\_ox, the EPR signal arising from the spin-interacting state is lost within a few milliseconds with no concomitant appearance of that for the reduced iron-sulfur center, as would be expected if electrons are transferred from the flavosemiquinone of TMADH\_2eq* to ETF\_ox to give enzyme possessing oxidized flavin and reduced 4Fe/4S center (11). On the basis of these results, it has been proposed that electrons are transferred from the iron-sulfur center of TMADH to ETF\_ox (11), but direct evidence has been lacking. The present work provides direct evidence that electron transfer to ETF takes place exclusively via the iron-sulfur center of TMADH and determines the intrinsic rate constant for electron transfer and dissociation constant using stopped-flow rapid mixing technique. The results are incorporated into a comprehensive kinetic mechanism for the reaction of TMADH.

MATERIALS AND METHODS

Enzyme Purification and Materials—Methylophilus methylotrophus W_3A_1 was grown and trimethylamine dehydrogenase was purified as described by Steenkamp and Mallinson (4), with the exception that the gel filtration step of the purification was performed using Sephacryl S-200 instead of Sephadex G-200. Enzyme concentration was determined from the 442 nm absorbance of oxidized enzyme using an extinction coefficient of 27.3 \text{mM}^{-1} \text{cm}^{-1} (3). Enzyme assay was performed as described by McIntire (12). ETF from M. methylotrophus W_3A_1 was obtained essentially as described by Steenkamp and Gallup (7) with the exception that again Sephacryl S-200 was used instead of Sephadex G-100. ETF as isolated was partially reduced, so it was oxidized with ferricenium hexafluorophosphate and then passed through a Sephadex G-25 column equilibrated with 50 mM, pH 7.0, phosphate buffer. The concentration of ETF was determined from the absorbance of the oxidized form at 438 nm, using a molar extinction coefficient of 11.3 \text{mM}^{-1} \text{cm}^{-1} (7). Phenylhydrazine-inactivated TMADH was prepared as de-
scribed by Kasprzak et al. (13, 14). The concentration of TMADH thus inactivated was determined using an extinction coefficient of 14.8 M−1 cm−1 at 442 nm (13, 14). Phosphate buffer was obtained from Sigma, and boric acid from J enneil Chemical Co. Sodium dithionite was obtained from Virginia Chemicals and phenylhydrazine from Eastman Kodak. Ferriencium hexafluorophosphate was prepared as described by Lehman et al. (15, 16). Titanium citrate was prepared according to Zehnder et al. (17) by anaerobic addition of a 1.9 M solution of TiCl3 in 2.0 M hydrochloric acid (purchased from Aldrich) to an appropriate volume of 0.1 M sodium citrate (purchased from Pierce), followed by adjustment of the pH to 7.0.

Static Experiments—Oxidized ETF was placed in an anaerobic cuvette, covered with a black cloth (to prevent incidental photooxidation), and made anaerobic by alternately evacuating and flushing with O2-free argon. Solutions of reduced TMADH were prepared by being made anaerobic as above, then titrated with either titanium citrate or sodium dithionite. Samples (1.0 ml) of reduced enzyme were removed through a serum stopper using a Hamilton syringe and placed in one side of an anaerobic split cell, which had been made anaerobic in advance by flushing with O2-free argon. Anaerobic oxidized ETF (1.0 ml) was placed in the other side of the split cell, and a spectrum was recorded using a Hewlett-Packard 8452A single beam diode array spectrophotometer. The two protein solutions were then mixed by tipping the split cell and a second spectrum recorded, the difference between the two representing the static spectral change associated with electron transfer from TMADH to ETF.

Kinetic Experiments—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. Anaerobic TMADH was prepared as above in a tonometer equipped with a ground joint for the dithionite titration syringe, a side arm cuvette, and a three-way stopcock valve with a male Luer connector, and reduced by titration with sodium dithionite to the desired level. ETF was made anaerobic as above and transferred to a syringe fitted with a three-way valve so that anaerobic buffer could be added to the syringe to change the ETF concentration by serial dilution. The concentrations of ETF were at least 5 times larger than that of TMADH to ensure pseudo first-order conditions in the experiments described. Kinetic transients obtained after mixing reduced TMADH with ETF were monitored as transmittance voltage and collected by a high speed A/D converter, then converted to absorbance changes by OLIS software. Time courses thus obtained were fitted to sums of exponentials using an iterative nonlinear least squares Levenberg-Marquardt algorithm (18), using the expression \( \Delta A(t) = \sum \Delta \alpha_i \exp(-k_i t) \) (with the floating variables \( \Delta \alpha_i \) and \( k_i \) representing the absorbance change and observed rate constant, respectively, for the nth kinetic phase).

RESULTS

The Spectral Change Associated with Reaction of Reduced TMADH with Oxidized ETF—The absorption spectra for oxidized, 2- and 3-electron reduced TMADH are shown in Fig. 1A, and the spectra for oxidized and semiquinone forms of ETF are shown in Fig. 1B. To determine the absorbance change associated with electron transfer from TMADH to ETF, the following experiments were performed. 1.0 ml of 8.2 M fully reduced TMADH (generated by titration with dithionite) was placed in one compartment of an anaerobic split cell, with 1.0 ml of 50 M ETFox in the other compartment. A spectrum was recorded, after which the two solutions were thoroughly mixed and a second spectrum recorded. The observed difference spectrum exhibits an absorbance increase in the 300–410 nm and 462–600 nm ranges and a decrease in the 410–462 nm range (Fig. 1C). To demonstrate that the spectral change is quantitatively consistent with the reduction of ETFox and the reoxidation of TMADH3eq in a 3:1 stoichiometry, the difference spectra for reoxidation of TMADH3eq and reduction of 3 equivalents of ETFox were generated from the spectra of Fig. 1 (A and B) and added. The difference spectrum thus obtained (Fig. 1E, dotted line) is very similar to the experimental difference spectrum (Fig. 1E, solid line), demonstrating that 1 equivalent of TMADH3eq reduced 3 equivalents of ETFox. To determine whether bisulfite (formed upon oxidation of dithionite) interferes with electron transfer between TMADH and ETF, TMADH was fully reduced with titanium citrate and mixed with ETFox in a split cell. The same result as dithionite-reduced TMADH was obtained (data not shown), indicating that bisulfite binding to the flavin of TMADH does not take place to an appreciable extent under the present experimental conditions and can be neglected.

Comparable results to those described above are obtained when only partially reduced enzyme was used. When 11 ml of 14 \( \mu \)M TMADH3eq in the non-spin-interacting state (generated by titration with TiIII-citrate to the level of 2 reducing equivalents/subunit at pH 7.0) is mixed with 10 ml of 42 M ETFox, the spectral change shown in Fig. 1D is observed and found to be quantitatively consistent with the reduction of ETFox and the reoxidation of TMADH3eq in the ratio of 2:1. When TMADH2eq in the spin-interacting state (generated by reduction with 1 equivalent of trimethylamine in the presence of 3 M tetramethyl ammonium chloride) is mixed anaerobically with ETFox at pH 7.0 in a split cell, the spectral change is again consistent with the reduction of ETFox and the reoxidation of the spin interacting state of TMADH2eq in the ratio 2:1. These results demonstrate that ETF is able to fully reoxidize TMADH.

Spectral Changes Associated with the Reaction of Reduced Phenylhydrazine-inactivated and Ferriencium-treated TMADH with Oxidized ETF—In addition to the above studies with native TMADH, we have examined the reoxidation of two covalently modified forms of TMADH by ETF. Inactivation of TMADH by reaction with phenylhydrazine results in addition of the phenyl moiety at the C(4a) position of the flavin to form a stable adduct (14). The absorption spectrum of 4a-phenylFMN resembles that of reduced flavin and the modified cofactor is redox-inert, so that when phenylhydrazine-inactivated TMADH is reduced with sodium dithionite the spectral change (Fig. 2A) is due entirely to reduction of the enzyme iron-sulfur center. When reduced, phenylhydrazine-inactivated enzyme is mixed anaerobically with ETFox in a split cell experiment of the type described above, the absorbance change shown in Fig. 2B is observed, exhibiting an absorbance increase in the 300–424 nm and 466–600 nm regions and an absorbance decrease in the 424–466 nm region. This spectral change is quantitatively consistent with the reduction of ETFox and the reoxidation of the reduced, inactivated TMADH in a stoichiometry of 1:1 (Fig. 2C). It is conceivable that the phenyl group of the inactivated enzyme dissociates in the course of this experiment, complicating the interpretation of the results. To demonstrate that this is not the case, phenylhydrazine-inactivated TMADH was reduced with dithionite, mixed with ETFox as above, and separated from the reaction mix by passage through a small Sephacryl S-200 column. It was found that the TMADH fraction consisted entirely of phenylhydrazine-inactivated enzyme, as determined both spectrophotometrically and by enzyme assay. We conclude that the reduced iron-sulfur center of phenylhydrazine-inactivated TMADH is able to reduce ETF even when the flavin center of the enzyme is rendered redox-inert by covalent modification. The difference spectrum for 4Fe4S center obtained from phenylhydrazine-inactivated TMADH, in agreement with that determined electrochemically (19), permits deconvolution of the difference spectrum for oxidized and fully reduced TMADH (Fig. 3).

Treatment of TMADH with 3 mM ferriencium hexafluorophosphate at pH 10 for 4 h at room temperature has been found empirically to give an iron-sulfur center that is EPR-active.2 The EPR spectrum of the enzyme thus generated is found to superficially resemble that given by the oxidized form of vari-
FIG. 1. Panel A, TMADH optical spectra. The spectra shown are for oxidized enzyme (solid line), 2-electron reduced enzyme (dashed line), and fully reduced enzyme (dotted line) at pH 7.0. Enzyme was reduced with titanium citrate to a level of 2 or 3 electrons/subunit. Panel B, ETF optical spectra. The spectra shown are for oxidized ETF (solid line) and dithionite reduced ETF (dotted line) in 50 mM potassium phosphate, pH 7.0. Panel C, optical spectra observed before and after mixing of fully reduced TMADH with oxidized ETF. Dithionite fully reduced TMADH (8.2 mM) was mixed anaerobically with 50 mM of oxidized ETF, pH 7.0, in a split cell (see "Materials and Methods"). The solid line is the spectrum recorded before mixing and the dotted line is the spectrum taken after mixing. Panel D, optical spectra observed before and after mixing of 2-electron reduced TMADH with oxidized ETF. TMADH (14 mM) reduced with titanium citrate to the level of 2 eq/subunit was mixed anaerobically with 42 mM oxidized ETF in 50 mM phosphate buffer, pH 7.0, in a split cell. The solid line is the spectrum recorded before mixing, and the dotted line is the spectrum taken after mixing. Panel E, theoretical and experimental difference spectra for the reaction of TMADHred with ETFox. The theoretical difference spectrum (dotted line) was obtained by adding the difference spectrum for reoxidation of 4.1 mM TMADHred (panel A) to the difference spectrum for reduction of 12.3 mM ETFox (panel B). The experimental difference spectrum (solid line) was obtained by subtracting the solid line in panel C from the dotted line in panel C.
ous high potential iron proteins (20). The integrated spin intensity of this signal indicates that the iron-sulfur center is quantitatively converted to this paramagnetic state. Since there was no loss of iron associated with generation of this EPR-active species (as one would expect if the signal arose from formation of a 3Fe/4S center), we tentatively conclude that the procedure results in the 1-electron oxidation of the iron-sulfur center to a level corresponding to that for oxidized high potential iron proteins. Regardless of the nature of this oxidation product, the significant aspect with regard to the present work is that the procedure renders the iron-sulfur center of TMADH redox-inert in that treatment with trimethylamine for 30 min does not reduce the intensity of the new EPR signal. It is found, however, that trimethylamine is still able to react with and reduce the enzyme FMN, but the reduced enzyme thus generated was not able to reduce ETF, even after prolonged incubation. These results demonstrate the flavin center of ferricenium-treated TMADH remains catalytically competent, but that rendering the iron-sulfur center redox-inert prevents reoxidation of the reduced flavin by ETF.

Kinetics of the Reaction of Phenylhydrazine-inactivated and Native TMADH with ETF—In order to further characterize the oxidative half-reaction of TMADH, the kinetics of the reaction of ETF with various forms of TMADH have been examined. In an effort to establish the intrinsic rate constant for the reaction of ETF with the reduced iron-sulfur center of TMADH, its reaction with the reduced phenylhydrazine-inactivated enzyme was first investigated. This form of TMADH possesses fully reduced iron-sulfur center and a redox-inert FMN so that it gives only a single reducing equivalent (from the iron-sulfur center) upon reoxidation by ETF. The reaction of reduced phenylhydrazine-inactivated TMADH with ETF at pH 7.0 exhibits two kinetic phases (Fig. 4A). The fast phase of the reaction

3 Dithionite can not fully reduce the iron-sulfur center of ferricenium-treated TMADH. When ferricenium-treated TMADH was reduced with dithionite, the intensity of the new EPR signal decreased only one half.
The enzyme concentration after mixing was 2.1 M, and the concentration of inactivated TMADH after mixing was 3.0 M. The fit of the data gives $k_{\text{lim}} = 173 \text{ s}^{-1}$ and $K_d = 10 \mu M$. The open squares represent the data for the reaction of reduced phenylhydrazine-inactivated TMADH with ETF. The concentration of inactivated TMADH after mixing was 3.0 $\mu M$. The fit of the data gives $k_{\text{lim}} = 173 \text{ s}^{-1}$ and $K_d = 16 \mu M$. The filled circles represent the data for the fast phase of TMADH$_{\text{ox}}$ in the spin-interacting state with ETF. The enzyme concentration after mixing was 2.3 $\mu M$. The fit of the data gives $k_{\text{lim}} = 149 \text{ s}^{-1}$ and $K_d = 39 \mu M$. The filled squares represent the data for the fast phase of the reaction of TMADH$_{\text{ox}}$ in non-spin-interacting state with ETF. The concentration of TMADH after mixing was 2.1 $\mu M$. The fit of the data gives $k_{\text{lim}} = 157 \text{ s}^{-1}$ and $K_d = 24 \mu M$.

The temperature dependence of the rate of electron transfer from the 4Fe/4S center of TMADH to ETF was studied by reacting dithionite-reduced phenylhydrazine-inactivated TMADH with ETF$_{\text{ox}}$ at 5, 15, 25, and 35 °C. The observed rate constants were temperature dependent and the Arrhenius plot is linear (not shown), giving activation energy of 12.8 kcal/mol.

The reaction of fully reduced native TMADH with ETF also exhibits two kinetic phases, although in this case the extent of the spectral change associated with the slow phase (approx. 20%) to the overall spectral change in all likelihood represents a side reaction, possibly the auto-reduction of ETF under the reaction conditions. The absorbance changes obtained from the stopped-flow experiments are identical, within experimental error, to those calculated for the static difference spectra (Fig. 6), indicating that there is no absorbance loss in the dead time of the stopped-flow apparatus. To investigate the ion strength effect on the intrinsic $k_{\text{lim}}$ and $K_d$ for the reaction of ETF with the fully reduced iron-sulfur center of TMADH, dithionite-reduced, phenylhydrazine-inactivated TMADH was mixed with ETF$_{\text{ox}}$ in 50 mM KPi, pH 7.0, buffer containing 0.2 M KCl in a stopped-flow apparatus. The double-reciprocal plot of $k_{\text{obs}}$ versus ETF concentration is linear (data not shown) and gives $k_{\text{lim}} = 125 \text{ s}^{-1}$ and $K_d = 16 \mu M$, not much different from the values obtained in the absence of KCl. The fact that ion strength has little effect on $k_{\text{lim}}$ and $K_d$ indicates that electrostatic forces do not play a particularly significant role in the formation and decay of $E_{\text{red}}$ETF$_{\text{ox}}$ complex.

The oxidative half-reaction of Trimethylamine Dehydrogenase

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4 Anaerobic ETF is slowly reduced by light in the absence of any reagent. The slow phase of the reaction of reduced phenylhydrazine-inactivated TMADH with ETF accounts for only 20% of the total spectral change, in addition, reduced phenylhydrazine-inactivated TMADH possesses only 1 electron/subunit and its reaction with ETF should not be biphasic. When reduced phenylhydrazine-inactivated TMADH reacts with ETF under aerobic conditions, the transients show only one phase. Thus, the slow phase in all likelihood represents a side reaction, probably the auto-reduction of ETF. By contrast, for the reaction of fully reduced native TMADH with ETF, the slow phase contributes 67% of the total spectral change, corresponding to approximately 2 equivalents removed from the enzyme, and can not be disregarded.
shows kinetic transients obtained on mixing TMADH$_{2eq}$ with ETFox (14 M) with ETF$_{ox}$ (42 μM) at pH 7.0 minus the spectrum recorded before mixing (see Fig. 1D). The closed circles represent the kinetic data obtained by mixing TMADH$_{2eq}$ (14 μM) with ETF$_{ox}$ (37 μM) in 50 mM phosphate buffer, pH 7.0, at 25°C in a stopped-flow apparatus. Panel B, the solid line is the static difference spectrum calculated from the spectrum taken after mixing of dithionite-reduced phenylhydrazine-inactivated TMADH (12 μM) with ETF$_{ox}$ (34 μM) in 50 mM phosphate buffer, pH 7.0, minus the spectrum recorded before mixing (see Fig. 2B). The closed circles represent the kinetic data obtained by mixing reduced phenylhydrazine-inactivated TMADH (12 μM) with ETF$_{ox}$ (36 μM) in 50 mM phosphate buffer, pH 7.0, in a stopped-flow apparatus at 25°C.

The reaction of ETF with reduced, phenylhydrazine-inactivated enzyme gives the intrinsic kinetic parameters for the reaction of reduced iron-sulfur center in TMADH$_{2eq}$ with ETF$_{ox}$ is dependent on whether the iron-sulfur center existed in a strong magnetic interaction with the flavin site. As in the case of fully reduced enzyme, the reaction of TMADH$_{2eq}$ in the non-spin-interacting state with ETF$_{ox}$ is biphasic (data not shown). The fast phase accounts for about 30% of the total absorbance change, and the observed rate constant is dependent on the ETF concentration; a double-reciprocal plot of $k_{obs}$ for the fast phase versus ETF concentration is linear (Fig. 5, filled circles), and the fit of the data gives $k_{lim} = 157 s^{-1}$ and $K_d = 24 μM$. The observed rate constant for the slow phase is approximately 10 s$^{-1}$ and is independent of [ETF] under pseudo-first-order conditions. In this experiment, approximately 40% of the TMADH$_{2eq}$ exists initially with an electron distribution possessing flavin semiquinone and reduced iron-sulfur center, but in which the two unpaired spins are not interacting to any detectable extent (22). When the experiment is repeated using TMADH$_{2eq}$ in the spin-interacting state (generated by reduction of the enzyme with 1 equivalent of trimethylamine in the presence of 3 mM tetramethyl ammonium chloride), the reaction again exhibits two kinetic phases (data not shown). The fast phase accounts for about 70% of the total absorbance change and the observed rate constant is ETF concentration dependent, with $k_{lim}$ and $K_d$ of 149 s$^{-1}$ and 39 μM, respectively (Fig. 5, filled circles). The rate constant for the slow phase, which accounts for approximately 30% of the total absorbance change, is about 4 s$^{-1}$ and independent of ETF concentration.

**DISCUSSION**

The present results indicate that when TMADH is treated with phenylhydrazine, rendering the FMN redox-inert (13), the iron-sulfur center can be reduced by dithionite and reoxidized by ETF. Similarly, when TMADH is treated with ferriencenium hexafluorophosphate at high pH, oxidizing the iron-sulfur center to a paramagnetic but redox-inert state, the FMN can be reduced into trimethylamine but cannot be reoxidized by ETF. These results strongly suggest that reducing equivalents introduced into TMADH at the flavin site in the course of turnover are transferred to ETF exclusively via the iron-sulfur center. This is consistent with the interpretation of previous kinetic results, which have also implicated the iron-sulfur center as the site of the oxidative half-reaction of TMADH (11).

The reaction of ETF with reduced, phenylhydrazine-inactivated enzyme gives the intrinsic kinetic parameters for the reaction of ETF with the fully reduced iron-sulfur center without the complication of subsequent electron transfer from the flavin and further reduction of ETF. The limiting rate constant of 173 s$^{-1}$ for electron transfer from the reduced iron-sulfur center to ETF is much faster than the rate-limiting step in the reduction reaction (product dissociation, with a rate constant of 3.5 s$^{-1}$; Ref. 9), so electron transfer from the iron-sulfur center of TMADH to ETF is not rate-limiting during steady-state turnover. The fact that both $k_{lim}$ and $K_d$ (16 μM) are insensitive to high salt concentration indicates that electrostatic forces are not involved in the formation and decay of E$_{ox}$ETF$_{ox}$ complex. The good agreement between both $k_{lim}$ and $K_d$ for the reoxidation of reduced, phenylhydrazine-inactivated TMADH with ETF$_{ox}$ and the fast phase of the reoxidation of...
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Fully reduced enzyme support the conclusion that the former reaction accurately represents the intrinsic reaction of enzyme possessing fully reduced iron-sulfur center with ETF, and that reaction of the enzyme flavin with phenylhydrazine does not significantly perturb the iron-sulfur center.

The fast phase of the reaction of TMADH$_{3eq}$ with ETF accounts for one third of the total absorbance change, and $k_{lim}$ is the same as that for the reaction of phenylhydrazine-inactivated TMADH with ETF, indicating the fast phase represents the removal of the first reducing equivalent from the reduced 4Fe/4S center to give TMADH$_{2eq}$. Because the slow phase accounts for two thirds of the total absorbance change, it most likely represents removal of the second and third equivalents from the FMNH$_2$ of TMADH$_{2eq}$ steps which are not kinetically resolved. Consistent with this interpretation is the good agreement between the rate constants for the slow phases of the reactions of ETF with TMADH$_{2eq}$ and TMADH$_{3eq}$. The distribution of the reducing equivalents favors the enzyme form with FMNH$_2$ and oxidized 4Fe/4S in TMADH$_{2eq}$ and with FMNH$_2$ and oxidized 4Fe/4S in TMADH$_{3eq}$. The reoxidation of fully reduced TMADH by ETF can thus be summarized as shown in Scheme 1.

The fast phases of the reactions of ETF with TMADH$_{2eq}$ in non-spin-interacting state and in spin-interacting state account for 30% and 70% of the total absorbance change, respectively, presumably because only 40% of the 4Fe/4S center in TMADH$_{2eq}$ in non-spin-interacting state and all the 4Fe/4S center in TMADH$_{2eq}$ in spin-interacting state are reduced. $k_{lim}$ for the fast phases of the two reactions are within experimental error (15–20%, Ref. 23). These rate constants for both slow phases are slightly faster than the rate-limiting steps (23). In the absence of tetramethylammonium chloride, the rate of reaction with ETF is identical, within experimental error, to that for the reaction of phenylhydrazine-inactivated TMADH with ETF. The small rate constants for both slow phases are owing to the unfavorable distribution of the reducing equivalents in TMADH$_{2eq}$ and TMADH$_{3eq}$. The distribution of the reducing equivalents favors the enzyme form with FMNH$_2$ and oxidized 4Fe/4S in TMADH$_{2eq}$ and with FMNH$_2$ and oxidized 4Fe/4S in TMADH$_{3eq}$. The reoxidation of fully reduced TMADH by ETF can thus be summarized as shown in Scheme 1.

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The rate constant for the slow phase (200 s$^{-1}$) is very rapid (50 μM). Following this initial rapid reaction, two slower kinetic phases (approximately 80 and 200 ms, respectively) are observed. The rate constant for the slowest phase is approximately equal to $k_{obs}$ (9–11, 24). By using the non-physiological substrate, diethylmethylamine, it has been demonstrated that product release and the binding of the second substrate molecule to the enzyme is rate limiting step (23). Intramolecular electron transfer within TMADH$_{3eq}$ has been studied using a pH jump technique and intramolecular equilibrium of reducing equivalents is fast ($k_{obs} \approx 200$ s$^{-1}$) (25).

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