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THE REACTION MECHANISM OF TRIMETHYLAMINE DEHYDROGENASE:
STRUCTURE AND FUNCTION RELATIONSHIPS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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1996

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ABSTRACT

1. The pH dependence of static optical/EPR spectra of trimethylamine dehydrogenase reduced to the level of two equivalents (TMADH$_{2eq}$) has been examined and indicates the existence of three different states for this iron-sulfur flavoprotein. At pH 6, TMADH$_{2eq}$ exists principally in a form possessing FMN hydroquinone, with its iron-sulfur center oxidized. At pH 8 the enzyme principally contains FMN semiquinone and reduced iron-sulfur, but despite the proximity of the two centers to one another, their magnetic moments do not interact. At pH 10, TMADH$_{2eq}$ exhibits the EPR spectrum that is diagnostic of a previously characterized spin-interacting state in which the magnetic moments of the flavin semiquinone and reduced iron-sulfur center are strongly ferromagnetically coupled. The kinetics of the interconversion of these three states has been investigated using a pH-jump technique in both H$_2$O and D$_2$O. The observed kinetic behavior is consistent with a reaction mechanism involving sequential protonation/deprotonation and intramolecular electron transfer events. All reactions studied show a normal solvent kinetic isotope effect. Proton inventory analysis indicates that at least one proton is involved in the reaction between pH 6 and pH 8, which principally controls intramolecular electron transfer, whereas at least two protons are involved between pH 8 and pH 10, which principally control formation of the spin-interacting state. The results of these and previous studies indicate that for TMADH$_{2eq}$, between pH 10 and pH 6, at least three protonation/deprotonation events are associated with intramolecular electron transfer and formation of the spin-interacting state, with
estimated $pK_a$ values of 6.0, 8.0, and -9.5. These $pK_a$'s are attributed to the flavin hydroquinone, flavin semiquinone and an undesignated basic group on the protein, respectively.

2. 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 ($k_{lim}$) 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 one-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 two-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 kinetic behavior of the oxidative half-reaction is 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.

3. The role played by the 6-S-cysteinyl FMN bond of trimethylamine dehydrogenase in the reductive half-reaction of the enzyme has been studied by following the reaction of the slow substrate diethylmethylamine with a C30A mutant of the enzyme lacking the covalent flavin attachment to the polypeptide. Removal of the 6-S-cysteinyl FMN bond diminishes the limiting rate for the first of the three observed kinetic phases of
the reaction by a factor of six, but has no effect on the rate constants for the two subsequent kinetic phases. The flavin in the C30A enzyme recovered from the reaction of C30A enzyme with excess substrate is found to have been converted to the 6-hydroxy derivative, rendering the enzyme inactive. The noncovalently bound FMN of the C30A mutant enzyme is also converted to 6-hydroxyFMN and rendered inactive upon reduction with excess trimethylamine, but not by reduction with dithionite, even at high pH or in the presence of the effector tetramethylammonium chloride. These results demonstrate that one significant role of the 6-S-cysteinyl FMN bond is to prevent the inactivation of the enzyme during catalysis. A reaction mechanism is proposed whereby OH\textsuperscript{-} attacks C(6) of a flavin-substrate covalent adduct in the course of steady-state turnover to form 6-hydroxyFMN.

4. Ferricenium-treated trimethylamine dehydrogenase (TMADH) has been characterized with UV/Vis absorption, CD, EPR, Mössbauer, and MCD spectroscopies. After treatment with ferricenium hexafluorophosphate at pH 10, a modified iron-sulfur center gives rise to an axial EPR signal with g-values of 2.04 and 2.01 which resembles the EPR spectra of \textit{Rhodopseudomonas gelatinosa} high potential iron protein (HiPIP) and of 3Fe cluster in beef liver cytoplasmic aconitase. The new EPR signal cannot be saturated by up to 140 mW microwave power. The modified iron-sulfur center cannot be converted back to a ferredoxin type 4Fe-4S center. The Mössbauer and MCD results show that the modified iron-sulfur center is neither a 3Fe cluster, nor a HiPIP 4Fe-4S center. Excess substrate trimethylamine can reduce the FMN, but not the modified iron-sulfur center in ferricenium-treated TMADH. When ferricenium-treated TMADH is reduced to one electron per subunit at pH 10, the modified iron-sulfur center cannot interact with flavin semiquinone, suggesting that the 4Fe-4S center is necessary for the formation of the spin-interacting state of native TMADH.
Dedicated to my parents
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<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>ETF</td>
<td>Electron transferring flavoprotein</td>
</tr>
<tr>
<td>ETF&lt;sub&gt;ox&lt;/sub&gt;</td>
<td>Oxidized Electron transferring flavoprotein</td>
</tr>
<tr>
<td>ETF&lt;sub&gt;sq&lt;/sub&gt;</td>
<td>Electron transferring flavoprotein semiquinone</td>
</tr>
<tr>
<td>4Fe/4S&lt;sub&gt;ox&lt;/sub&gt;</td>
<td>Oxidized four iron-four sulfur center</td>
</tr>
<tr>
<td>4Fe-4S&lt;sub&gt;red&lt;/sub&gt;</td>
<td>Reduced four iron-four sulfur center</td>
</tr>
<tr>
<td>FMN&lt;sub&gt;sq&lt;/sub&gt;</td>
<td>Flavin mononucleotide semiquinone</td>
</tr>
<tr>
<td>HiPIP</td>
<td>High potential iron protein</td>
</tr>
<tr>
<td>MCD</td>
<td>Magnetic Circular dichroism</td>
</tr>
<tr>
<td>TMAC</td>
<td>Tetramethylammonium chloride</td>
</tr>
<tr>
<td>TMADH</td>
<td>Trimethylamine Dehydrogenase</td>
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<td>Trimethylamine dehydrogenase reduced to the level of two equivalent per subunit</td>
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<td>TMADH&lt;sub&gt;2eq*&lt;/sub&gt;</td>
<td>Spin-interacting state of trimethylamine dehydrogenase</td>
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<tr>
<td>TMADH&lt;sub&gt;3eq&lt;/sub&gt;</td>
<td>Fully reduced trimethylamine dehydrogenase</td>
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CHAPTER 1

INTRODUCTION

1.1 Trimethylamine Dehydrogenase

Trimethylamine dehydrogenase (TMADH; EC 1.5.99.7) purified from the restricted facultative methylotroph *Methylophilus methylotrophus* (previous referred to as W3A1) catalyzes the oxidative demethylation of trimethylamine to dimethylamine and formaldehyde and subsequently transfers electrons one at a time to its natural electron acceptor, electron transferring flavoprotein (ETF). TMADH is a homodimer with molecular weight of 166,000 Da. Each subunit contains a covalently bound FMN, a ferredoxin-type 4Fe-4S center, and an ADP of unknown function. The enzyme is the first protein found in which the enzyme-bound FMN is covalently linked to the protein via a 6-S-cysteinyl FMN bond (1-10). Throughout this dissertation, one subunit will be referred to as one enzyme. The gene encoding TMADH has been cloned (11) and the amino acid sequence of TMADH deduced from the DNA sequence of TMADH gene is shown in Fig. 1.1.

The crystal structure of TMADH has been solved at 2.4 Å resolution (Fig. 1.2) (12, 13). Each subunit of TMADH is folded into three domains. The large N-terminal domain, composed of residues 1-383 and containing the FMN and 4Fe-4S center, is folded as an eight-stranded parallel b barrel surrounded by eight parallel a helices. The
Fig. 1.1 DNA sequence of TMADH gene and the deduced amino acid sequence. (taken from ref. 11)
Fig. 1.2 Crystal structure of TMADH
medium domain which includes residues 384-494 and 649-733 is folded as a five-stranded parallel β sheet flanked by two helices on one side and by three on the other side. The small domain, containing residues 495-648, is also folded as a five-stranded parallel β sheet, but flanked by three parallel helices on one face and by three-stranded anti-parallel β sheet on the other (12). ADP is located between the medium and small domains (13).

The active site of TMADH is shown in Fig. 1.3. The FMN is covalently linked via its C(6) position to residue Cys-30. The flavin ring is nonplanar. It bends 20° about the N(5) to N(10) axis of the flavin ring. It is located at the bottom of a 14 Å deep channel and is accessible to solvent. The 4Fe-4S center is bound to four cysteine residues: Cys-345, Cys-347, Cys-351, and Cys-364. The distance between the centers of the FMN and 4Fe-4S center is approximately 12 Å. But the 8a-methyl group of FMN is 6 Å away from the closest iron atom and 4 Å from the cysteinyl sulfur atom of Cys-351 (12, 13).

In addition to trimethylamine, dimethylamine, triethylamine, and diethylamine are substrates of TMADH (10). More chemicals have been determined to be the substrates for TMADH from bacterium 4B6 (14, 15). These substrates are listed as follows. Tertiary amines: 2-hydroxyethyldiethylamine, 2-chloroethyldimethylamine, ethylidimethylamine, diethylmethylamine, 2-aminoethyldimethylamine, 2-dimethylamino-methylpropanol, dimethylaminoacetonitrile; secondary amines: ethylmethylamine, O, N-dimethylhydroxylamine. Primary, quaternary, poly-, and diamines are not substrates. TMADH can be inhibited by diethylamine, acetaldehyde, ethylamine, dimethylamine, tetramethylammonium chloride, phenylhydrazine and tri(hydroxymethyl)-aminomethane buffer (10, 15-17). TMADH from bacterium 4B6 can also be inhibited by Cu^{2+}, Co^{2+}, Ni^{2+}, Hg^{2+}, Ag^{2+}, iodoacetamide, N-ethylmaleimide, p-chloromercuribenzoate, trimethylsulfonium chloride (14). The irreversible inhibition of TMADH by "suicide inhibitors" of monoamine oxidase, phenylhydrazine and a variety of
Fig. 1.3 The active site of TMADH
other alkyl and arylhydrazines, results in the alkylation or arylation of the flavin at the C(4a) position and thus inactivation of the enzyme (14, 18).

The reduction potentials of the two prosthetic groups in TMADH have been determined in the absence and presence of tetramethylammonium chloride (19-22) and are listed in Table 1.1.

When TMADH is reduced with dithionite, each subunit takes up three reducing equivalents and a rhombic EPR signal with g-values of 1.84, 1.93, and 2.03 for reduced 4Fe-4S center is observed. By contrast, when reduced with substrate, TMADH can take up only two reducing equivalents to form flavin semiquinone and reduced 4Fe-4S center. The unpaired electron spins of the flavin semiquinone and the reduced 4Fe-4S center interact to give a spin-interacting species which exhibits absorption maxima at 365, 430, and 510 nm, a complex EPR signal at g=2, and an unusually strong half-field EPR signal at g~4. Excess of trimethylamine is required for maximal formation of the spin-interacting state at pH 7, but not at pH>8.5. On the other hand, in the presence of tetramethylammonium chloride, one equivalent of trimethylamine is sufficient for the formation of the spin-interacting state. These observations have been interpreted as indicating that the formation of the spin-interacting state is modulated by the binding of substrate or a substrate analog. In addition, reduction of the enzyme with dithionite in the presence of tetramethylammonium chloride results in the formation of the spin-interacting state, not the fully reduced enzyme (4, 8, 18). It has been shown that only a single binding site exists by gel chromatography of reduced TMADH with [14C] trimethylamine (16). One mol of reduced TMADH binds 0.87 mol of [14C] trimethylamine. To explain the effect of excess substrate and tetramethylammonium chloride on the formation of spin-interacting species, it has been proposed that the reduction potentials and/or the locations of the FMN and 4Fe-4S center are changed by the binding of tetramethylammonium chloride to the enzyme (4, 16). It has been
Redox couple FMN/FMN$_{sq}$ [4Fe-4S]$^{2+}$/[4Fe-4S]$^{1+}$ FMN$_{sq}$/FMNH$_2$ ref.

<table>
<thead>
<tr>
<th>pH</th>
<th>Direction</th>
<th>V</th>
<th>V</th>
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<tr>
<td>7.0$^a$</td>
<td>Reductive</td>
<td>+0.24</td>
<td>+0.05</td>
<td>-0.05</td>
</tr>
<tr>
<td></td>
<td>Oxidative</td>
<td>+0.23</td>
<td>+0.07</td>
<td>-0.04</td>
</tr>
<tr>
<td>8.4</td>
<td>Reductive</td>
<td>+0.23</td>
<td>+0.04</td>
<td>-0.18</td>
</tr>
<tr>
<td></td>
<td>Oxidative</td>
<td>+0.23</td>
<td>+0.04</td>
<td>-0.12</td>
</tr>
<tr>
<td>7.0</td>
<td>Reductive</td>
<td>+0.044</td>
<td>+0.102</td>
<td>+0.036</td>
</tr>
<tr>
<td>8.5</td>
<td>Reductive</td>
<td>-0.008</td>
<td>+0.070,-0.0043$^b$</td>
<td>-0.029</td>
</tr>
<tr>
<td>9.1</td>
<td></td>
<td>-0.020$^c$</td>
<td>+0.065</td>
<td></td>
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a. Data for TMADH in the presence of tetramethylammonium chloride.
b. Two values were obtained for the 4Fe-4S center.
c. Redox potentials for two-electron reduction of FMN.

Table 1.1 Reduction potentials of trimethylamine dehydrogenase
demonstrated that the reduction potentials of TMADH are changed in the presence of tetramethylammonium chloride (22). However, studies of crystalline TMADH in three oxidation states and in the presence of substrate and inhibitor tetramethylammonium chloride show that ligands bind at the active site and that the positions of the FMN and 4Fe-4S center are unchanged (23).

Steady-state kinetic studies of TMADH have shown that the enzymatic reaction proceeds via a ping-pong mechanism. The double-reciprocal plot of initial velocity versus the concentration of one substrate at different fixed concentrations of the second substrate consists of a set of parallel straight lines (10). Steady-state analysis of TMADH using both trimethylamine and phenazine methosulfate as substrates in 0.1 M sodium pyrophosphate at pH 7.7 and 10°C gives $k_{cat}$ of 3.09 s$^{-1}$ (24). The apparent $K_m$ for trimethylamine in the presence of 2 mM phenazine methosulfate is 10 mM (18). When ETF is used as an oxidant, the dependence of reaction rate on ETF concentration at a fixed concentration of trimethylamine shows simple saturation kinetics with an apparent $K_m$ of 6.7 mM, although trimethylamine inhibits the reaction in the millimolar range (25).

The reductive half-reaction of TMADH has been studied using trimethylamine or diethylmethylamine as substrate in a stopped-flow apparatus (4, 18, 24, 26). The reaction of TMADH with trimethylamine exhibits three kinetic phases: a rapid phase followed by two slower phases. The fast phase has been shown to represent the two-electron reduction of the flavin by substrate. This phase is complete within a few ms at pH 7.7 and 10 °C and is too fast to be rate-limiting in catalysis. The double-reciprocal plot of the observed rate constants for the fast phase versus trimethylamine concentration passes through the origin, indicating that the reaction is second order. The second-order rate constant is $68.6 \times 10^4$ M$^{-1}$s$^{-1}$ at 10 °C. The intermediate and the slow phases have half lives of about 80 ms and 200 ms, respectively, at pH 7.7 and 18 °C. It is impossible to determine if these two phases are simultaneous or consecutive because they are not well
separated in time. These two slower phases coincide roughly with the formation of the spin-interacting species determined by freeze-quench EPR experiments and have been interpreted as representing intramolecular electron transfer from the flavin hydroquinone to the iron-sulfur center (4, 18, 24). However, pH-jump studies of intramolecular electron transfer within two-electron reduced TMADH have shown that intramolecular electron transfer is one hundred-fold faster than the reductive half-reaction and is in no way rate-limiting (27). It seems likely instead that binding of substrate and product to TMADH modulates the intramolecular electron transfer.

When trimethylamine is used as a substrate for the kinetic study of the reductive half-reaction of TMADH, the fast phase is too fast to be seen in a stopped-flow apparatus at high concentration of trimethylamine (>500 mM) and the two slower phases are not well separated. In order to solve these difficulties, diethylmethylamine, a slow substrate, is used instead of trimethylamine and an overall reductive half-reaction kinetic mechanism has been proposed (26) which is shown in Fig. 1.4. The reaction of TMADH with diethylmethylamine also exhibits three kinetic phases. During the fast kinetic phase, substrate binds to free enzyme reversibly to form a Michaelis complex. A general base abstracts a proton from the substrate to form a substrate carbanion which then adds to the N(5) position of the flavin ring to form a flavin-substrate covalent adduct. During the intermediate phase, the flavin-substrate covalent adduct is broken down to form flavin hydroquinone which then transfers one reducing equivalent to oxidized 4Fe-4S center. The slow phase represents the dissociation of the product followed by the binding of a second substrate molecule to fully develop the spin-interacting state (26).

The FMN in TMADH is covalently attached to residue Cys-30 via a 6-S-cysteinyl FMN bond. When Cys-30 is replaced with alanine by site-directed mutagenesis, each subunit of the C30A mutant TMADH contains one 4Fe-4S center and one ADP, but only 30% of the C30A mutant enzyme consists of FMN and the FMN is bound noncovalently.
Fig. 1.4 Reductive half-reaction mechanism for TMADH (taken from ref. 26). Enzyme species inside boxes are spectroscopically identifiable and correspond to oxidized enzyme and reaction intermediates at the conclusion of the fast kinetic phase, intermediate phase and slow phase, respectively.
The C30A mutant is found to also catalyze the demethylation of trimethylamine. For C30A mutant enzyme, the apparent $K_m$ for trimethylamine is one hundred-fold larger than that seen with native TMADH while the apparent $k_{cat}$ is one-half of that observed with native TMADH. When C30A mutant enzyme is reduced with dithionite in the presence of 2 mM tetramethylammonium chloride, the noncovalently bound FMN is reduced to flavin semiquinone level only. Similar to native TMADH, the unpaired electron spins of the noncovalently bound flavin semiquinone and reduced 4Fe-4S center interact to give a spin-interacting state which exhibits a complex $g=2$ EPR signal and a strong $g\sim4$ EPR signal (28).

Both recombinant wild-type and C30A mutant TMADH purified from *E. coli* are a mixture of holo- and deflavo enzyme (28). The deflavo enzyme cannot be reconstituted by incubation with excess FMN in the presence of KBr, a method used to reconstitute many flavoproteins (29). However, noncovalently bound FMN in C30A enzyme can be removed by dialysis against 1 M KBr and rebinds to the deflavo enzyme thus generated in the presence of KBr (28). On the other hand, the H29C, C30H double mutant TMADH does not contain any flavin. Electrospray mass spectrometry studies demonstrate that the *in vivo* synthesized deflavo enzyme is not post-translationally modified. Thus, it appears most likely that binding of FMN probably occurs during the folding of the enzyme. The *in vivo* deflavo enzyme may be misfolded and is unable to recognize FMN (30).

### 1.2 Electron Transferring Flavoprotein

ETFs transfer electrons from enzymes of mitochondrial and bacterial degradation pathways to their respiratory electron transport chains. ETF purified from *Methylophilus methylotrophus* is the natural electron acceptor of TMADH. ETF is a heterodimer with molecular weight of 77,000 and contains a noncovalently bound FAD and an AMP (25,
31). The genes encoding the two subunits of ETF have been cloned and sequenced (32). The sequence of the genes and the deduced amino acid sequences are shown in Fig. 1.5. It has been proposed that the FAD and AMP bind to the large subunit (32). ETF has been crystallized and the crystal structure is under investigation (33).

TMADH in the presence of trimethylamine reduces ETF to the anionic semiquinone form which is stable in air. Dithionite and deazaflavin mediated photoreduction also fail to fully reduce ETF (25). However, ETF can be fully reduced to the hydroquinone form electrochemically. The reduction potentials for the formation of semiquinone (ETF$_{\text{ox}}$/ETF$_{\text{sq}}$) and the second electron reduction (ETF$_{\text{sq}}$/ETF$_{\text{red}}$) are +196 mV and -197 mV, respectively (35). The first reduction potential (+196 mV) is pH independence and the most positive reduction potential seen for FAD-containing proteins. The second potential (-197 mV) is much larger than those for dithionite (-530 mV) and deazaflavin radical (-650 mV), indicating that the prevention of the enzymatically and chemically full reduction of ETF is due to a kinetic rather than a thermodynamic barrier. Thus, ETF cycles between fully oxidized and semiquinone forms rather than between fully oxidized and fully reduced forms during catalysis (25, 34–36).

The oxidative half-reaction of TMADH has been studied by the freeze-quench technique using both phenazine methosulfate (14) and ETF (24) as electron acceptor. When substrate-reduced TMADH is mixed with phenazine methosulfate or ETF, the EPR signals arising from the spin-interacting state disappear within a few ms, indicating that the reoxidation of reduced TMADH by phenazine methosulfate or ETF is not rate-limiting in the course of catalysis.
The sequence of the genes and the deduced amino acid sequences of ETF. The amino acid residues that were confirmed by NH₂-terminal sequencing of the purified subunits and the PCR primer regions are underlined. Termination codons are indicated by an asterisk. Arrows locate potential stem-loop structures.
1.3 General Description of the Thesis Research

When TMADH is reduced with substrate, the FMN in TMADH first takes up two reducing equivalents from substrate and subsequently transfers one to the 4Fe-4S center to form flavin semiquinone and reduced 4Fe-4S center. The magnetic moments of the flavin semiquinone and the reduced 4Fe-4S center interact to give a spin-interacting state which exhibits a complex $g=2$ EPR signal and a strong $g\sim 4$ half-field signal. The spin-interacting state is involved in catalysis. Previous study shows that the spin-interacting state can also be generated by reduction of TMADH with dithionite to the level of two reducing equivalents per subunit at high pH. Both the distribution of reducing equivalents between the FMN and the 4Fe-4S center in partially reduced enzyme and the formation of the spin-interacting state are pH-dependent. Several questions arise from the known behavior of the enzyme. Why is the spin-interacting state formed at high pH, but not at low pH? How many protons are involved in the intramolecular electron transfer between the two prosthetic groups and in the formation of the spin-interacting state? What are the $pK_a$ values for those protonation/deprotonation steps? These are addressed in Chapter 2 of the thesis. In Chapter 2, reductive optical/EPR titrations of TMADH are described that examine redox states of the two prosthetic groups at different pH and the $pK_a$ values associated with the intramolecular electron transfer and the formation of the spin-interacting state. Because the absorption spectrum of partially reduced enzyme is dependent on pH, the kinetics of intramolecular electron transfer and the formation of the spin-interacting state are studied in both $H_2O$ and $D_2O$ using a pH jump technique. A mechanism involving sequential protonation/deprotonation and intramolecular electron transfer steps is then proposed.

So far, most of the mechanistic studies of TMADH have focused on the reductive half-reaction and the intramolecular electron transfer. Little has been done on the
oxidative half-reaction. The studies of the reductive half-reaction have demonstrated that the FMN rather than the 4Fe-4S center accepts reducing equivalents from substrate, but the site interacting with ETF has not been determined. In Chapter 3, experiments are carried out to demonstrate that reducing equivalents are transferred solely from the 4Fe-4S center of TMADH to ETF. In addition, the kinetics of the oxidative half-reaction is investigated in a stopped-flow apparatus using ETF and various forms of TMADH.

Another important question has to do with why the FMN is covalently linked in TMADH. Can the enzyme function normally without the 6-S-cysteiny1 FMN bond? In Chapter 4, the role of the 6-S-cysteiny1 FMN bond in catalysis is investigated using C30A mutant TMADH lacking the covalent flavin attachment to the polypeptide.

Finally the 4Fe-4S cluster of TMADH has been examined spectroscopically. 4Fe-4S centers can exist in three oxidation states: [4Fe-4S]$^{3+}$, [4Fe-4S]$^{2+}$, and [4Fe-4S]$^{1+}$, but any given protein will use only two of these. The bacterial ferredoxin contains [4Fe-4S]$^{2+}$ in the oxidized form and [4Fe-4S]$^{1+}$ in the reduced form, while oxidized and reduced high potential proteins (HiPIP) contain [4Fe-4S]$^{3+}$ and [4Fe-4S]$^{2+}$, respectively. So far, attempts of conversion of ferredoxin-type [4Fe-4S]$^{2+}$ into high potential iron protein (HiPIP) [4Fe-4S]$^{3+}$ have not succeeded. TMADH contains a ferredoxin-type [4Fe-4S]$^{2+}$, can it be converted to HiPIP-type [4Fe-4S]$^{3+}$? In Chapter 5, an EPR signal similar to that for HiPIP-type [4Fe-4S]$^{3+}$ is generated by treating TMADH with ferricenium hexafluorophosphate at high pH. The ferricenium-treated TMADH is then further characterized using UV/Vis absorption, circular dichroism (CD), EPR, Mössbauer, and magnetic circular dichroism (MCD) spectroscopies.
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CHAPTER 2

PROTOTROPIC CONTROL OF INTRAMOLECULAR ELECTRON TRANSFER IN TRIMETHYLAMINE DEHYDROGENASE

2.1 Abstract

The pH dependence of static optical/EPR spectra of trimethylamine dehydrogenase reduced to the level of two equivalents (TMADH_{2eq}) has been examined and indicates the existence of three different states for this iron-sulfur flavoprotein. At pH 6, TMADH_{2eq} exists principally in a form possessing FMN hydroquinone, with its iron-sulfur center oxidized. At pH 8 the enzyme principally contains FMN semiquinone and reduced iron-sulfur, but despite the proximity of the two centers to one another, their magnetic moments do not interact. At pH 10, TMADH_{2eq} exhibits the EPR spectrum that is diagnostic of a previously characterized spin-interacting state in which the magnetic moments of the flavin semiquinone and reduced iron-sulfur center are strongly ferromagnetically coupled. The kinetics of the interconversion of these three states have been investigated using a pH-jump technique in both H_{2}O and D_{2}O. The observed kinetics are consistent with a reaction mechanism involving sequential protonation/deprotonation and intramolecular electron transfer events. All reactions studied show a normal solvent kinetic isotope effect. Proton inventory analysis indicates
that at least one proton is involved in the reaction between pH 6 and pH 8, which principally controls intramolecular electron transfer, whereas at least two protons are involved between pH 8 and pH 10, which principally control formation of the spin-interacting state. The results of these and previous studies indicate that for TMADH$_{2eq}$, between pH 10 and pH 6, at least three protonation/deprotonation events are associated with intramolecular electron transfer and formation of the spin-interacting state, with estimated $pK_a$ values of 6.0, 8.0, and $\sim$9.5. These $pK_a$'s are attributed to the flavin hydroquinone, flavin semiquinone and an undesignated basic group on the protein, respectively.
2.2 Introduction

Trimethylamine dehydrogenase (TMADH; EC 1.5.99.1) as isolated from bacterium W3A1 is a 166 kDa iron-sulfur flavoprotein. The enzyme consists of two identical, catalytically independent subunits, each containing a covalently bound 6-cysteiny1 FMN coenzyme and a 4Fe/4S (bacterial ferredoxin-type) iron-sulfur center (1-4). During turnover, trimethylamine dehydrogenase is reduced by trimethylamine at the FMN site (7-9) and is believed to be oxidized at the iron-sulfur center, in vivo, by an electron-transferring flavoprotein (5-6; L. Huang and R. Hille, unpublished). Intramolecular transfer of reducing equivalents from FMN to the iron-sulfur center thus appears to be a necessary step in the catalytic cycle. Full reduction of trimethylamine dehydrogenase requires the uptake of three reducing equivalents, two for full reduction of the FMN, and the third for reduction of the iron-sulfur center. When reduced to the level of two equivalents per active site, an equilibrium distribution of reducing equivalents between the two centers exists which is dictated by the relative reduction potentials of the FMN and 4Fe/4S center. Two possible electron distributions within enzyme reduced by only two equivalents (TMADH2eq) exist: (1) flavin hydroquinone and oxidized iron-sulfur center; and (2) flavin semiquinone and reduced iron-sulfur center. The latter distribution has been seen with trimethylamine dehydrogenase reduced by excess substrate, with enzyme reduced by sodium dithionite in the presence of the inhibitor tetramethylammonium chloride, and with dithionite reduced enzyme at pH 10 (6-11). In each case, interaction of the magnetic moments of the unpaired electrons of the flavin semiquinone and reduced iron-sulfur center gives rise to a spin-interacting state which is distinguished by a complex EPR signal centered near $g = 2$ and an unusually intense half-field ($g \sim 4$) signal (12). It has been shown that this spin-interacting state forms in the absence of any substantive protein conformational changes (13).
Reductive optical/EPR titrations of trimethylamine dehydrogenase with sodium dithionite have shown that the equilibrium distribution of reducing equivalents between the FMN and 4Fe/4S center in partially reduced enzyme is dependent on pH (11). Formation of fully reduced flavin with oxidized iron-sulfur is favored at low pH whereas formation of flavin semiquinone and reduced iron-sulfur is preferred at high pH. The absorption spectrum of partially reduced enzyme exhibits changes that reflect this pH-dependence, making it is possible to follow the kinetics of intramolecular electron transfer with a stopped-flow rapid mixing apparatus by using a pH-jump technique (11). In these experiments, partially reduced enzyme is prepared in a weakly buffered (e.g. 10 mM) solution at a known initial pH and then rapidly mixed with a strongly buffered (e.g. 100 mM) solution at a different final pH. The initial equilibrium distribution of reducing equivalents within the enzyme (determined by the initial pH) is thus rapidly perturbed, permitting the kinetics of the approach of the system to the new equilibrium position (determined by the final pH) to be followed spectrophotometrically (14).

Both electron transfer (from the flavin hydroquinone to the oxidized iron-sulfur center) and formation of the spin-interacting state occur in the course of the catalytic cycle of trimethylamine dehydrogenase. The present studies have been undertaken to further examine the role of protonation/deprotonation events in each of these two processes. We find that the behavior of the enzyme is best described by a model in which electron transfer and protonation/deprotonation events are treated as independent equilibria. In addition, evidence is presented that formation of the spin-interacting state of trimethylamine dehydrogenase is governed by an ionization having a pK\textsubscript{a} of ~9.5.

2.3 Materials and Methods

*Enzyme purification and materials.* Bacterium W3A\textsubscript{1} was grown and trimethylamine dehydrogenase was purified as described by Steenkamp and Mallinson.
(15), with the exception that the gel filtration step was performed using Sephacryl S-200 instead of Sephadex G-200. Phosphate and pyrophosphate buffers were obtained from Sigma and boric acid from Jennielle Chemical Co. Use of inorganic buffers (i.e. phosphate, pyrophosphate, borate) is preferred over organic buffers in studies of trimethylamine dehydrogenase since most organic buffers contain substituted amines which either inhibit the enzyme or serve as substrates. Benzyl viologen and phenazine ethosulfate were also obtained from Sigma. Sodium dithionite was obtained from Virginia Chemicals. Concentrated sodium dithionite stock solutions were prepared by addition of the solid to an anaerobic buffer solution which was transferred to an anaerobic syringe equipped with a ground glass joint. D₂O (99.9% enriched) was obtained from Cambridge Isotope Laboratories. Isotopic mixtures of enzyme and buffer solutions were prepared by mixing appropriate volumes of separately buffered solutions of H₂O and D₂O, with the D₂O solutions prepared taking into account the necessary correction of the pH meter reading to obtain the pD (pD = meter reading + 0.4; ref. 16). pD for D₂O buffers was adjusted using either NaOD (40% w/w in 99.9% D₂O) or DCl (20% w/w in 99.9% D₂O), both obtained from Cambridge Isotope Laboratories. D₂O solutions were prepared immediately before use and kept in capped bottles sealed with Parafilm® to prevent contamination with atmospheric moisture.

Preparation of TMADH₂eq and D₂O solutions. In order to prepare two-electron reduced trimethylamine dehydrogenase for pH(D)-jump measurements, concentrated samples of oxidized enzyme were passed through a Sephadex G-25 column (1.5 x 25 cm) equilibrated with a 10 mM solution of an appropriate buffer, and adjusted to the desired initial pH. Enzyme in D₂O was obtained by passage through a separate Sephadex G-25 column equilibrated with D₂O buffer (initial swelling of the dried gel material having also been performed with D₂O). 10 mM buffers adjusted to the initial pH(D) also contained 0.1 M potassium chloride, 0.5 μM phenazine ethosulfate, and 0.5 μM benzyl viologen. Potassium chloride was included to maintain solvent ionic strength throughout
the experiment. The dyes were included to facilitate initial enzyme reduction and, at the low concentrations used, have been shown to have no effect on the observed kinetics (11). Solutions of 0.1 M buffer at the desired mole fraction of D$_2$O and desired final pH(D) were added to 20 ml glass syringes, bubbled for 30 minutes with anhydrous, O$_2$-free argon, and sealed with rubber septa. For experiments in mixed solvent, enzyme was diluted with the appropriate buffer to give the desired mole fraction D$_2$O and a final enzyme concentration of 50 - 100 $\mu$M (25 - 50 $\mu$M dimers) and placed with a tonometer equipped with a ground glass joint for the dithionite titration syringe, a side arm cuvette and a three-way stopcock valve with a male Luer connector. The sample was made anaerobic by repeated evacuation and flushing with anhydrous, O$_2$-free argon, after which the tonometer was fitted with an anaerobic syringe containing a dithionite solution and the enzyme titrated to the level of two equivalents per subunit with sodium dithionite. No differences were observed between enzyme solutions prepared in D$_2$O and used immediately and enzyme solutions prepared in D$_2$O and allowed to stand overnight before use.

Earlier studies have shown that the bisulfite product of dithionite oxidation reacts with oxidized trimethylamine dehydrogenase at pH $\leq$ 7 (11). As an alternative to dithionite, low pH enzyme samples used in optical/EPR measurements were reduced with titanium citrate solutions (see below). This method of reduction is inappropriate for the preparation of pH-jump samples, however, since titanium citrate requires strongly buffered (0.1 - 0.2 M) citrate solutions to remain in solution. The bisulfite adduct problem was avoided in the earlier pH-jump study by first reducing the enzyme in 1 mM borate buffer, pH 10, and then bringing the solution to 10 mM phosphate at the desired initial pH by the addition of concentrated phosphate buffer (11). However, for the purposes of this study, the complexity of this procedure would likely lead to H$_2$O contamination of D$_2$O solutions and was not used. Since formation of the bisulfite
adduct renders the FMN redox-inert, any enzyme molecules which have reacted with bisulfite (estimated to be no greater than 10-20% in any case) are assumed not to contribute to the absorbance change on the timescale of the pH(D)-jump experiment and merely add to the total background absorbance. Any substantial breakdown of a sulfite adduct under the present experimental conditions would be expected to give rise to extremely slow spectral changes. The absence of such slow spectral changes in the transients observed in the course of the work presented here indicates that sulfite adduct formation presents a negligible problem in the present studies.

Rapid reaction studies. Rapid reaction studies were carried out as described previously, using a stopped-flow apparatus whose calibrated dead-time was determined to be 600 μs (11). Time courses were fitted to either single or double exponential expressions (ΔA(t) = ΣΔA_n exp(-k_n t)) using an iterative non-linear least squares Levenberg-Marquardt algorithm (17) for the parameters ΔA_n and k_n representing the total absorbance change and observed rate constant, respectively, exhibited by the nth kinetic phase.

Optical/EPR titrations. Optical/EPR titrations were performed using an anaerobic cuvette equipped with a quartz observation cell and a port sealed with a rubber septum. Anaerobic samples of oxidized enzyme were prepared at the desired pH through the use of a Sephadex G-25 column and titrated with reductant as described above. For samples at pH ≥ 8, sodium dithionite was used as the reductant; enzyme samples at pH < 8 were reduced with titanium citrate, prepared according to Zehnder et al. (18) by anaerobic addition of a 1.9 M solution of TiCl₃ in 2.0 M hydrochloric acid (from Aldrich) to an appropriate volume of 0.1 M sodium citrate (from Pierce), followed by adjustment of the pH to 7.0. After reduction to an appropriate level, 400 μl enzyme samples were removed using a long-needle Hamilton syringe and placed in quartz EPR tubes which had been previously flushed with O₂-free argon and finally frozen on liquid nitrogen. X-band EPR
spectra were recorded at 15 K using a Bruker ER 300 EPR spectrometer equipped with a ER035M gaussmeter and a Hewlett-Packard 5352B microwave frequency counter. Instrument parameters are given in the appropriate figure legends. A total of 20-50 40-second scans were accumulated for each sample to improve the signal-to-noise ratio. EPR signal intensities due to flavin semiquinone and reduced iron sulfur were determined by double integration of spectra using Bruker Instruments software. Intensities of the half-field signal indicative of the spin-interacting state were determined using the peak-to-trough amplitude of the signal at g ~ 4.

2.4 Results

*pH Dependence of TMADH*<sub>2eq</sub> *Optical/EPR Spectra* - Optical/EPR reductive titrations of trimethylamine dehydrogenase at pH 6 and pH 10 have established the pH dependence of the intramolecular electron distribution between the FMN and 4Fe/4S centers in partially reduced enzyme (11). It has also been shown that the FMN semiquinone seen in partially reduced enzyme ionizes over this pH range: the neutral form is observed at pH 6 and the anionic form at pH 10. The visible absorbance spectrum for TMADH*<sub>2eq</sub> at pH 10 exhibits absorbance peaks at 365, 440, and 510 nm (Fig 2.1, ‒ ‒ ‒ ), and is nearly identical to that seen at pH 8.0 with enzyme reduced by excess substrate, or by dithionite in the presence of the inhibitor tetramethylammonium chloride (7, 8, 11). Previous work has shown that this three-banded spectrum arises from the form of the enzyme which exhibits the complex high-field EPR spectrum and half-field (g ~ 4) signal indicative of the spin-interacting state (Fig. 2.2, A and B; refs. 5 - 12). Since these EPR features reflect magnetic interaction of the unpaired electron spins on the two centers, TMADH*<sub>2eq</sub> at pH 10 must possess (anionic) flavin semiquinone and a reduced iron-sulfur center. At pH 6, on the other hand, the reductive titration data (11) indicate that much of the flavin
Figure 2.1. *pH dependence of TMADH_{2eq} optical spectra.* The spectra shown are for oxidized enzyme at pH 7.0 (upper solid line), enzyme reduced by dithionite to 2 eq per subunit at pH 10.0 (-- -- --), pH 8.0 (-----), and by titanium citrate at pH 6.0 (· · · · · ·), and enzyme fully reduced by dithionite at pH 7.0 (lower solid line). The two-electron reduced sample at pH 10.0 was prepared by titration with dithionite solution in 0.1 M borate buffer, pH 10.0, containing 0.5 mM benzyl viologen. The two-electron reduced sample at pH 8.0 was prepared by titration with dithionite to the level of 2 eq per subunit in 0.1 M sodium pyrophosphate, pH 8.0, containing 0.5 mM benzyl viologen. The two-electron reduced sample at pH 6.0 was prepared by titration with titanium citrate solution in 0.1 M potassium phosphate buffer, pH 6.0, containing 0.5 mM benzyl viologen. Titanium citrate was used to prevent the formation of bisulfite-TMADH complexes at low pH. The kinetically determined absorption spectrum of the first intermediate formed in the course of the reaction of trimethylamine dehydrogenase with diethylmethylamine, exhibiting the spectrum of reduced flavin and oxidized iron-sulfur center, is shown (open circles) for comparison (11).
Figure 2.2. *EPR spectra of TMADH$_{2eq}$ at pH 10.0 and 8.0.* Enzyme in 0.1 M potassium borate, pH 10.0 or 0.1 M sodium pyrophosphate, pH 8.0 buffer containing 0.5 mM benzyl viologen was reduced with dithionite to 2 eq per subunit. EPR parameters: microwave frequency, 9.44955 GHz; microwave power, 1.00 milliwatts; modulation amplitude, 10.084 gauss; 15 K. Panel A is the high field region of the spectrum of TMADH$_{2eq}$ at pH 10.0. Panel B is the half-field region of the spectrum of TMADH$_{2eq}$ at pH 10.0. Panel C is the high field region of the spectrum of TMADH$_{2eq}$ at pH 8.0. Panel D is the half-field region of the spectrum of TMADH$_{2eq}$ at pH 8.0.
Figure 2.2 (continued)
exists as the hydroquinone in TMADH\textsubscript{2eq} (Fig 2.1, \ldots). At no time during the pH 6 reductive titration are the EPR features characteristic of the spin-interacting state observed.

The visible absorbance spectrum of TMADH\textsubscript{2eq} at pH 8 is intermediate between that seen at pH 6 and 10, showing some of the features present in the pH 10 spectrum and suggesting that two-electron reduced enzyme contains mostly flavin semiquinone and reduced iron-sulfur center at pH 8 (Fig. 2.1, - - -). However, the features at 365 and 440 nm are not as well defined at pH 8 as they are at pH 10 and the peak at 510 nm seen in the pH 10 spectrum is virtually absent in the spectrum at pH 8, indicating that a significant difference exists in TMADH\textsubscript{2eq} at these two pH values. These differences appear to reflect changes in ionization of the flavin semiquinone and, to a lesser degree, a shift in the electron distribution toward further iron-sulfur reduction at the higher pH.

Differences between TMADH\textsubscript{2eq} at pH 8 and 10 is most pronounced when examined by EPR. At pH 10 the EPR spectrum of TMADH\textsubscript{2eq} reflects formation of the spin-interacting state (Fig. 2.2, Panels A and B). At pH 8 however, the high-field region of the EPR spectrum reflects a simple combination of flavin semiquinone and reduced 4Fe/4S signals (Fig. 2.2C) and no half-field feature indicative of spin-interaction is observed (Fig. 2.2D). EPR spectra of samples at pH 8 were recorded at several microwave power levels in order to facilitate quantitation of these signals (the flavin semiquinone readily power saturates at the low temperatures required to observe the reduced 4Fe/4S center). Spin integration of the axial flavin semiquinone and rhombic 4Fe/4S EPR signals for TMADH\textsubscript{2eq} at pH 8 extrapolated to very low microwave power (< 1 µW, so as not to saturate the flavin semiquinone signal) confirms a 1:1 stoichiometry, \textit{i.e.}, an electron distribution in which one reducing equivalent is on the FMN and one is on the iron-sulfur center. This important result indicates that an intramolecular electron distribution
consisting of a flavin semiquinone and a reduced iron-sulfur center is necessary, but not sufficient, to produce the spin-interacting form of the enzyme.

One or more ionizable groups with $pK_a$ values $> 8$ must be responsible for induction of the spin-interacting state in TMADH$_{2eq}$ on raising the pH from 8 to 10. The pH-dependence of the $g \sim 4$ signal intensity at 15 K is shown in Figure 3A, and indicates that formation of the spin-interacting state of the enzyme is controlled by a single ionizable group exhibiting a $pK_a$ of 9.4. In an effort to correlate formation of the spin-interacting state with ionization of the neutral flavin semiquinone and to determine directly the $pK_a$ for ionization of the semiquinone, the pH dependence of the absorbance at 365 nm of TMADH$_{2eq}$ has been examined. The anionic form of flavin semiquinone displays an absorbance band at this wavelength (19), and the case of trimethylamine dehydrogenase is relatively uncomplicated by the spectral contribution of its iron-sulfur center. The pH dependence of the 365 nm absorbance in TMADH$_{2eq}$ is shown in Figure 3B, where it is evident that the absorbance grows in over too great a pH range to be attributable to a single ionization. A fit of the data using a two-$pK_a$ equation suggests that full formation of the semiquinone anion requires the ionizations of two groups exhibiting $pK_a$ values of 8.0 and 9.7, the latter being within experimental error of that determined from the pH dependence of the $g \sim 4$ signal. One of these ionizations must derive from the N(5) position of the flavin semiquinone while the other presumably derives from another site in the protein, possibly hydrogen-bonded or otherwise associated with the iron-sulfur center. The data indicate that three conditions must be met for formation of the spin-interacting state in TMADH$_{2eq}$: (1) the distribution of reducing equivalents within the active site must give flavin semiquinone and reduced iron-sulfur center; (2) the semiquinone must be ionized; and (3) a third group within the active site must also be deprotonated. All of these requirements are met at pH 10, but only the first (and possibly the second, to a greater or lesser extent) is met at pH 8.
Figure 2.3. *pH dependence of EPR half-field signal intensity and extinction coefficient at 365 nm*. Panel A, relative g=4 signal intensity is plotted *versus* the pH at which the experiments were performed. The filled circles represent the amount of g=4 signal observed at a given pH, normalized to the amount seen at pH 10.0. The solid line represents a fit of the data to a single pKₐ expression, yielding a value of 9.4. Panel B, the maximum extinction coefficient at 365 nm (filled circles) during reductive titration of TMADH with dithionite (at pH ≥8.0) or titanium citrate (at pH <8.0) is plotted *versus* pH. The filled circles represent the extinction coefficients. The solid line represents a fit of the data to a double pKₐ expression, yielding values of 8.0 and 9.7.
Fig. 2.3

\[ \varepsilon \text{ (mM}^{-1}\text{ cm}^{-1}) \]

\[ g = 4 \text{ signal intensity} \]
**pH(D)-Jump Kinetics.** [High pH] minus [low pH] kinetic difference spectra for TMADH$_{2eq}$ show maxima at 365, 410, and 520 nm (11) that agree well with the static absorbance changes reported in Figure 2.1. This indicates that it should be possible to separately monitor intramolecular electron transfer and formation/decay of the spin-interacting state of TMADH$_{2eq}$ subsequent to a pH jump. Accordingly, pH(D)-jump experiments between pH(D) 8 and 6 (following principally intramolecular electron transfer between the flavin and iron-sulfur center), between pH(D) 10 and 8 (principally following formation of the spin-interacting state), and between pH(D) 10 and 6 (which monitors both processes) have been undertaken. Time courses for the 10 → 6 and 10 → 8 pH-jump reactions with TMADH$_{2eq}$, as carried out by stopped-flow in H$_2$O, exhibit monophasic behavior and are successfully fit using a single exponential expression (Fig. 2.4, A and B). The kinetic behavior and fitted rate constants obtained are consistent with the results of previous work (11), with $k_{obs} = 440$ s$^{-1}$ and 420 s$^{-1}$ for the 10 → 6 and 10 → 8 pH-jump reactions, respectively (all rate constants with standard deviations are summarized in Table 2.1). These values are independent of observation wavelength within the uncertainty associated with these measurements.$^4$ In D$_2$O, the 10 → 6 pD-jump reaction also exhibits well-behaved monophasic kinetics with $k_{obs} = 56$ s$^{-1}$ (Fig. 2.4C). A comparison of the observed rate constants in H$_2$O and D$_2$O yields an observed solvent kinetic isotope effect of 7.9 for the pH(D) 10 → 6 reaction.

By contrast to the well-behaved monophasic behavior exhibited by the reactions described above, the 10 → 8 pD-jump reaction with TMADH$_{2eq}$ in D$_2$O exhibits complex kinetic behavior (Fig. 2.4D). The time courses show a distinct lag phase at all three wavelengths monitored, followed by monophasic kinetic behavior. Ignoring the lag phase, rate constants for these time courses have been obtained by fitting the data points collected at times ≥ 20 ms after mixing to a single exponential expression. The results (Fig 2.4D, solid lines) give an observed rate constant for the 10 → 8 pD-jump reaction of
Figure 2.4. *Time courses observed for the TMADH<sub>2eq</sub> pH(D)-jump reaction in 100% H<sub>2</sub>O and 100% D<sub>2</sub>O.* Samples of two-electron reduced trimethylamine dehydrogenase were prepared, and stopped-flow rapid mixing experiments were performed as described in "Material and Methods". The final enzyme concentrations range from 50 to 100 μM. Absorbance changes observed at 365, 410, and 520 nm after mixing are plotted versus time. The *solid lines* represent fits of the data to exponential expressions. The values of the observed rate constants for each wavelength shown in the figure represent the average of at least six independent measurements at each wavelength for a given set of conditions. 

*Panel A*, pH 10 → 6 (100% H<sub>2</sub>O). Data are fitted to a single exponential expression. 

*Panel B*, pH 10 → 8 (100% H<sub>2</sub>O). Data are fitted to a single exponential expression. 

*Panel C*, pD 10 → 6 (100% D<sub>2</sub>O). Data are fitted to a single exponential expression. 

*Panel D*, pD 10 → 8 (100% D<sub>2</sub>O). Data collected at time t ≥ 20 ms are fitted to a single exponential expression in order to account for the lag phase. *Panel E*, pH 6 → 10 (100% H<sub>2</sub>O). Data are fitted to a single exponential expression. 

*Panel F*, pH 8 → 10 (100% H<sub>2</sub>O). Data are fitted to a single exponential expression. *Panel G*, pD 6 → 10 (100% D<sub>2</sub>O). Data are fitted to a double exponential expression and the fraction of absorbance change attributed to each kinetic phase is given next to the value of the observed rate constant. *Panel H*, pD 8 → 10 (100% D<sub>2</sub>O). Data are fitted to a double exponential expression and the fraction of absorbance change attributed to each kinetic phase is given next to the value of the observed rate constant. *Panel I*, pH 8 → 6 (100% H<sub>2</sub>O). Data are fitted to a single exponential expression. *Panel J*, pH 6 → 8 (100% H<sub>2</sub>O). Data are fitted to a single exponential expression. *Panel K*, pD 8 → 6 (100% D<sub>2</sub>O). Data are fitted to a single exponential expression. *Panel L*, pD 6 → 8 (100% D<sub>2</sub>O). Data are fitted to a single exponential expression. *Panel M*, pD 8 → 6 (100% D<sub>2</sub>O) at 520 nm. The kinetic transient was collected at two time scales: 0 - 20 ms and 20 - 200 ms. Data are fitted to a double exponential expression.
Fig. 2.4

A. pH 10 → pH 6

- 365 nm
  - $k_{\text{obs}} = 430 \text{ s}^{-1}$

- 520 nm
  - $k_{\text{obs}} = 440 \text{ s}^{-1}$

B. pH 10 → pH 8

- 365 nm
  - $k_{\text{obs}} = 420 \text{ s}^{-1}$

- 520 nm
  - $k_{\text{obs}} = 450 \text{ s}^{-1}$

- 410 nm
  - $k_{\text{obs}} = 400 \text{ s}^{-1}$
Figure 2.4 (continued)

C. pD10 → pD 6

365 nm
\[ k_{\text{obs}} = 56 \text{ s}^{-1} \]

520 nm
\[ k_{\text{obs}} = 54 \text{ s}^{-1} \]

410 nm
\[ k_{\text{obs}} = 57 \text{ s}^{-1} \]

D. pD 10 → pD 8

365 nm
\[ k_{\text{obs}} = 59 \text{ s}^{-1} \]

520 nm
\[ k_{\text{obs}} = 59 \text{ s}^{-1} \]

410 nm
\[ k_{\text{obs}} = 62 \text{ s}^{-1} \]
Figure 2.4 (continued)

E. pH 6 → pH 10

\[ \Delta A_t \] vs. Seconds

410 nm
\[ k_{obs} = 890 \text{ s}^{-1} \]

520 nm
\[ k_{obs} = 920 \text{ s}^{-1} \]

F. pH 8 → pH 10

365 nm
\[ k_{obs} = 970 \text{ s}^{-1} \]

410 nm
\[ k_{obs} = 990 \text{ s}^{-1} \]

365 nm
\[ k_{obs} = 1000 \text{ s}^{-1} \]

520 nm
\[ k_{obs} = 1000 \text{ s}^{-1} \]
Figure 2.4 (continued)

G. pH 6 → pH 10

410 nm
\[ k_1 = 310 \text{ s}^{-1} (55\%) \]
\[ k_2 = 47 \text{ s}^{-1} (45\%) \]

365 nm
\[ k_1 = 320 \text{ s}^{-1} (54\%) \]
\[ k_2 = 76 \text{ s}^{-1} (46\%) \]

520 nm
\[ k_1 = 290 \text{ s}^{-1} (45\%) \]
\[ k_2 = 64 \text{ s}^{-1} (55\%) \]

H. pH 8 → pH 10

410 nm
\[ k_1 = 550 \text{ s}^{-1} (36\%) \]
\[ k_2 = 65 \text{ s}^{-1} (64\%) \]

365 nm
\[ k_1 = 260 \text{ s}^{-1} (57\%) \]
\[ k_2 = 45 \text{ s}^{-1} (43\%) \]

520 nm
\[ k_1 = 260 \text{ s}^{-1} (46\%) \]
\[ k_2 = 47 \text{ s}^{-1} (54\%) \]
Figure 2.4 (continued)

For pH 8 → pH 6:
- 520 nm, \( k_{\text{obs}} = 250 \text{ s}^{-1} \)

For pH 6 → pH 8:
- 410 nm, \( k_{\text{obs}} = 400 \text{ s}^{-1} \)
- 520 nm, \( k_{\text{obs}} = 400 \text{ s}^{-1} \)
Figure 2.4 (continued)

- For 365 nm, $k_{\text{obs}} = 190 \text{ s}^{-1}$.
- For 410 nm, $k_{\text{obs}} = 120 \text{ s}^{-1}$.
- For 520 nm, $k_{\text{obs}} = 93 \text{ s}^{-1}$.

Graphs showing the changes in absorbance ($\Delta A_t$) over time (Seconds) for different wavelengths and pH changes.
Figure 2.4 (continued)

M. pD 8 → pD 6 520 nm

\[ k_1 = 170 \text{ s}^{-1} \]
\[ k_2 = 23 \text{ s}^{-1} \]
<table>
<thead>
<tr>
<th>pH(D) jump</th>
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<th>520 nm</th>
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<tr>
<td></td>
<td>$k_{fast}$</td>
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<td>s$^{-1}$</td>
<td>s$^{-1}$</td>
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<td>440 ± 30 (wavelength-independent)</td>
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<td>200 ± 19</td>
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<td>190</td>
<td>120 ± 8</td>
<td>170</td>
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Table 2.1. *Rate constants observed in pH(D) jump experiments with TMADH$_{2eq}$.* Rate constants and associated errors are given for each of the experiments described in the present study. In the case of rate constants in excess of 500 s$^{-1}$, the standard deviation obtained from fits of a given transient (typically $\sim$10% of the magnitude of the fitted rate constant) does not accurately reflect the uncertainty in the measurement from one transient to the next at a given condition. This uncertainty is estimated to be closer to 50%.
60 s\(^{-1}\). Comparison with the corresponding rate constant in H\(_2\)O gives an observed solvent kinetic isotope effect of 7.0 for the pH(D) 10 \(\rightarrow\) 8 reaction. The emergence of the lag phase in the 10 \(\rightarrow\) 8 pH(D)-jump time courses as the mole fraction of D\(_2\)O present in the solvent increases (not shown) suggests a multi-step mechanism for loss of the spin-interacting state on jumping the pH(D) from 10 to 8.

pH jump experiments in the reverse direction (i.e. pH(D) 6 \(\rightarrow\) 10 and pH(D) 8 \(\rightarrow\) 10) have also been performed. When performed in H\(_2\)O, the results are consistent with those obtained from the previous trimethylamine dehydrogenase pH-jump study (11), with observed time courses that exhibit fitted rate constants of 1000 s\(^{-1}\) for the 8 \(\rightarrow\) 10 reaction and 930 s\(^{-1}\) for the 6 \(\rightarrow\) 10 reaction. Again, well-behaved monophasic time courses are observed, with wavelength-independent rate constants (Fig. 2.4, E and F). In D\(_2\)O, however, the observed time courses are distinctly biphasic (Fig. 2.4, G and H), and a two-exponential expression is required to fit both the 6 \(\rightarrow\) 10 and 8 \(\rightarrow\) 10 pH-jump time courses satisfactorily (solid lines, Fig. 2.4, G and H). For the 6 \(\rightarrow\) 10 pH-jump reaction, the rate constant for the fast kinetic phase is 310 s\(^{-1}\) (which contributes approximately half of the total observed absorbance change at all three wavelengths monitored). The rate constant obtained for the slow kinetic phase of the reaction, on the other hand, is wavelength-dependent, ranging from 76 s\(^{-1}\) at 365 nm to 47 s\(^{-1}\) at 410 nm. This behavior suggests that the slow phase of the reaction consists of multiple components having distinct spectral changes, but which are kinetically unresolved. Multiphasic kinetics makes the determination of an observed solvent kinetic isotope effect for the pH(D) 6 \(\rightarrow\) 10 jump work problematic, but the effect is clearly significant, in the range of 3 to 15 (depending on whether rate constants from the fast or slow phase of the reaction in D\(_2\)O are used in the calculation). As in the case for the D\(_2\)O 10 \(\rightarrow\) 8 pH-jump experiment in D\(_2\)O, the multiphasic behavior exhibited by the 6 \(\rightarrow\) 10 pH-jump reaction suggests a complex reaction mechanism.
The $8 \rightarrow 10$ pD-jump reaction also gives biphasic and wavelength-dependent time courses (Fig. 2.4H). At 365 and 520 nm, the fast phase of the reaction gives a rate constant of 260 s$^{-1}$ and accounts for about half of the total absorbance change observed at these wavelengths; the slow kinetic phase gives a rate constant of 46 s$^{-1}$. At 410 nm the observed rate constant is 550 s$^{-1}$, significantly larger than those observed at 365 and 520 nm; the rate constant for the slow kinetic phase of the reaction is 65 s$^{-1}$. Again, the implication is that the spectral change associated with each of the two kinetic phases of the reaction consist of multiple components. Calculating the solvent kinetic isotope effect for the $8 \rightarrow 10$ pH(D)-jump reaction is again complicated by the multiphasic kinetics observed in D$_2$O, but is in the range of 4 to 15.

For the $8 \rightarrow 6$ and $6 \rightarrow 8$ pH-jump experiments in H$_2$O, the results are significantly different than is observed in the other pH-jump experiments. In each of the other cases (pH $8 \rightarrow 10$, $10 \rightarrow 8$, $6 \rightarrow 10$ and $10 \rightarrow 6$) the time courses are monophasic and observed rate constants wavelength-independent for a given reaction. Wavelength-dependent rate constants are observed only in the biphasic low-to-high pH(D)-jump time courses in D$_2$O (Fig. 2.4G and H). In the case of the $8 \rightarrow 6$ pH-jump in H$_2$O, simple monophasic behavior is observed but the observed rate constants are wavelength-dependent, ranging from 500 s$^{-1}$ at 365 nm to 200 s$^{-1}$ at 410 nm (Fig. 2.4I). For the $6 \rightarrow 8$ pH-jump reaction, time courses at 410 and 520 nm are monophasic with an observed rate constant of 400 s$^{-1}$ (Fig. 2.4J). At 365 nm, however, no kinetic absorbance change is observed, although an increase is expected from the static difference spectrum (Fig 2.1). It appears that the spectral change at this wavelength occurs so rapidly that the absorbance change is lost in the 600μs dead time of the apparatus. Again, the implication is that there are multiple components to the overall spectral change associated with the reaction.

In all of the pH(D)-jump experiments performed up to this point, absorbance decreases at 365 and 520 nm are observed along with an absorbance increase at 410 nm.
when the pH(D) is decreased and the reverse observed when the pH(D) is increased (Fig. 2.4; ref. 11). In both the \(8 \rightarrow 6\) and \(6 \rightarrow 8\) pD-jump experiments in D\(_2\)O, by contrast, the direction of absorbance change at 365 nm is in the same direction as that seen at 410 nm (Fig. 2.4, K and L), and opposite to that observed observed at 365 nm in the corresponding H\(_2\)O experiment, at 520 nm the transient exhibits rise-fall behavior (Figure 2.4M). For the \(8 \rightarrow 6\) pD-jump reaction, the 365 nm kinetic transients collected at intermediate D\(_2\)O mole fractions exhibit rise-fall behavior, with contributions from absorbance changes in both directions (not shown). For the \(6 \rightarrow 8\) pH(D)-jump reaction a discernible absorbance change is observed at 365 nm only in 100% D\(_2\)O, and is too small to permit a precise evaluation of the observed rate constant (Fig. 2.4L).

A multi-step kinetic scheme must be invoked to account for the above observations. It appears that absorbance changes at 365 nm due to very rapid reactions which are lost in the dead time when performed in H\(_2\)O become slow enough to observe in D\(_2\)O. As the mole fraction of D\(_2\)O increases, two distinct kinetic processes are observed with absorbance changes in opposite directions at both 365 and 520 nm. Given the nature of the experiments, the data most likely reflect a protonation/deprotonation event (which is too rapid to observe in H\(_2\)O), followed by subsequent intramolecular electron transfer.

Proton Inventory of the Solvent Kinetic Isotope Effect - It is possible to determine the dependence of a rate constant as a function of the solvent mole fraction D\(_2\)O and plot the parameter \(k_{\text{obs}}/k_{\text{D}_2\text{O}}\) versus mole fraction of D\(_2\)O (22, 23). The shape of such "proton inventory" plots are described by the Gross-Butler equation (24, 25) and it is possible in principle to determine the exact number of protons involved in the reaction mechanism in this way. The simplest distinction is made between a reaction mechanism involving a single proton, which yields a linear plot, or multiple protons, in which case the plot is bowed. However, the precision in experimental data required to make such a
determination can be prohibitive (22), increasing as the overall solvent kinetic isotope effect decreases or as one tries to distinguish between mechanisms involving one- and two-, two- and three-, three- and four-proton etc.

A proton inventory analysis for the pH jump experiments with TMADH2eq is straightforward only for the 10 → 6 pH(D)-jump reaction since this is the only case where the time courses exhibit monophasic kinetics and wavelength-independent observed rate constants at all solvent mole fractions of D2O. In order to obtain rate constants for the 10 → 8 pH(D)-jump reaction, it is necessary to ignore a plainly visible lag phase from the kinetic transients (Fig. 2.4D). In the case of the 8 → 6 and 6 → 8 pH(D)-jump reactions, a proton inventory is possible if only the data obtained at 410 and 520 nm are considered. Finally, the 6 → 10 and 8 → 10 pH(D)-jump reactions display increasingly biphasic time courses as the solvent mole fraction of D2O increases, and neither the determination of an overall solvent kinetic isotope effect nor a proton inventory analysis is justified for these reactions.

With these limitations in mind, a proton inventory analysis has been performed for the 10 → 6, 10 → 8, 8 → 6, and 6 → 8 pH(D)-jump reactions (Fig. 2.5). The proton inventory plot for the 8 → 6 pH(D)-jump reaction is linear, consistent with the involvement of only a single proton in the reaction (Fig. 2.5A, circles); the overall observed solvent kinetic isotope effect is 1.7. The proton inventory plot for the pH(D)-jump in the reverse direction (6 → 8) is also linear, with an observed overall solvent kinetic isotope effect of 3.2. While the relative error in these stopped-flow rapid mixing experiments, defined as the standard deviation of the mean of at least six determinations, is too great to discriminate between one- and two-proton mechanisms, it is clear that both reactions involve at least one proton.

For both the 10 → 6 and 10 → 8 pH(D)-jump reactions, the proton inventory plots are distinctly convex downward (Fig. 2.5B). In both cases the precision of the kinetic data
Figure 2.5. Proton inventory analysis of TMADH$_{2eq}$ pH(D)-jump reactions. The observed rate constant divided by the rate constant observed in 100% D$_2$O is plotted versus the mole fraction of D$_2$O present in the solvent. The error bars represent the standard deviation of the mean of at least six independent measurements for a given set of conditions. Panel A, pH(D) 8 $\rightarrow$ 6 (filled circles) and pD 6 $\rightarrow$ 8 (filled triangles). The solid lines represent fits of the data to a linear expression. Panel B, pH(D) 10 $\rightarrow$ 6 (filled squares) and pD 10 $\rightarrow$ 8 (filled diamonds). The solid lines represent fits of the data to the Gross-Butler equation (see ref. 22 and references contained therein).
are sufficient to conclude that a minimum of two protons are involved (22). This interpretation is consistent with the static optical/EPR titration results (see above), which suggest that there are at least two ionizable groups which exhibit $pK_a$ values $> 8$ and are important in controlling formation or breakdown of the spin-interacting state. The precision of the kinetic data is not high enough, however, to distinguish between two- and three-proton reaction mechanisms. Taken together, the proton inventory data indicate that there is at least one proton involved in the reaction mechanism between pH(D) 6 and pH 8, and at least two protons involved in the reaction mechanism between pH(D) 8 and 10. Thus there must be at least three protons involved in the overall pH(D)-jump reaction mechanism between pH(D) 6 and 10.

2.5 Discussion

The pH-dependence of the static visible and EPR spectra from this and previous studies indicate that three identifiable states of TMADH$_{2eq}$ are most easily observed at pH 6, 8, and 10. At pH 6, TMADH$_{2eq}$ appears to consist principally in the form possessing flavin hydroquinone and oxidized iron-sulfur center. This is consistent with the $pK_a$ value of 6 estimated for the N(1) position of flavin hydroquinone from reductive half-reaction studies (26). At pH 8, TMADH$_{2eq}$ possesses mainly flavin semiquinone and reduced iron-sulfur center, but the magnetic moments of the unpaired spins do not interact at this pH. It is likely that pH 8 is close enough to the $pK_a$ value for the neutral/anionic flavin semiquinone equilibrium that significant amounts of both forms are present. Finally, at pH 10, trimethylamine dehydrogenase reduced by two equivalents possesses anionic flavin semiquinone and reduced iron-sulfur center, with the magnetic moments of the unpaired spins interacting strongly.
The data describe a situation in which the relative reduction potentials of FMN and the iron-sulfur center are such that an intramolecular electron distribution consisting of flavin hydroquinone and oxidized iron-sulfur is preferred at low pH, with the flavin hydroquinone predominantly protonated at the N(1) position of the isoalloxazine ring. Recent work on the reductive half-reaction of trimethylamine dehydrogenase with the alternative substrate diethylmethylamine suggests that the N(1) position of the flavin hydroquinone exhibits a pK_a value of approximately 6 (26). This value is comparable to the pK_a value for the N(1) position of free flavin hydroquinone which has been shown to exhibit a pK_a value near 6.5 (27). This value is reasonably expected to be lowered if the protein structure causes strain that introduces a bend in the flavin about a line connecting atoms N(5) and N(10) of the isoalloxazine ring, as is known to be the case for trimethylamine dehydrogenase (28). As the pH increases, loss of this proton to produce flavin hydroquinone anion apparently decreases the reduction potential for the hydroquinone/semiquinone couple below that for the iron-sulfur center, so that by pH 8.0 the intramolecular electron distribution favors flavin semiquinone and reduced iron-sulfur center over the distribution consisting of anionic hydroquinone and oxidized iron-sulfur by a factor of approximately four (26). This distribution is reflected in the EPR spectrum of trimethylamine dehydrogenase reduced by two equivalents at pH 8, which appears to be a simple combination of an axial flavin semiquinone radical signal and a rhombic signal due to reduced iron-sulfur center (Fig. 2.2C). At pH 8, the FMN semiquinone appears to be present mostly in the neutral form, as judged by the linewidth of its EPR signal. A further increase in pH leads to ionization at the N(5) position of the flavin semiquinone (which exhibits a pK_a of 8.0) to give the semiquinone anion. The semiquinone anion is unable to become reduced by back electron transfer without uptake of a proton (to do so would give rise to the very unstable dianionic flavin hydroquinone), and at sufficiently high pH the distribution shifts further in favor of iron-sulfur reduction.
over formation of the flavin hydroquinone. The static optical/EPR titration and stopped-flow rapid mixing kinetic data support the existence of still another ionizable group within trimethylamine dehydrogenase which exhibits a \( pK_a \) of approximately 9.5 (Fig. 2.3). Deprotonation of this ionizable group as the pH is increased from 8 to 10 results in interaction of the magnetic moments of the unpaired spins present on the flavin semiquinone anion and reduced iron-sulfur center. Thus at sufficiently high pH TMADH\(_{2eq}\) is predominantly in the form giving rise to the spin-interacting state, as evidenced by the EPR signal exhibited by the enzyme (Fig. 2.2, A and B).

Low pH favors an electron distribution in TMADH\(_{2eq}\) in which the flavin is largely reduced and iron-sulfur center oxidized, whereas high pH favors formation of the flavin semiquinone and reduced iron-sulfur center. This is consistent with the expected pH dependence of the three reduction potentials of the system, and with the observed pH dependence of the intensity of the half-field EPR signal (attributable to the spin-interacting state in which the iron-sulfur center must be reduced) and the intensity of the absorbance at 365 nm (attributable to the anionic flavin semiquinone) that is observed with TMADH\(_{2eq}\). The semiquinone form of FMN can exist in either the neutral (protonated) or anionic (deprotonated) form depending on the ionization state of the N(5) position of the isoalloxazine ring. Trimethylamine dehydrogenase has been shown to be unusual among flavoproteins in that it can accommodate either of these forms of flavin semiquinone depending on solvent pH, with a \( pK_a \) of approximately 8.0 (Fig. 2.3B; ref. 11). Similarly, the hydroquinone of flavin can exist as either the neutral or anionic form depending on the ionization state of the N(1) position. The ionization of N(1) of the hydroquinone has been inferred from the pH dependence of the reductive half-reaction of trimethylamine dehydrogenase with diethylmethylamine (26). The reduction potentials of both the partially and fully reduced forms of FMN increase with decreasing solvent pH since protonation of the isoalloxazine ring neutralizes a negatively charged electron. In
the simplest formulation, the possible pH dependence of the iron-sulfur center reduction potential has not been considered. The reduction potential of the iron-sulfur center might also be expected to increase with decreasing solvent pH for the same reason (e.g. protonation of a site near the 4Fe/4S cluster is expected to increase the reduction potential). However, in determining the pH dependence of TMADH$_{2eq}$ intramolecular electron distribution, it is the relative reduction potentials of the two centers which are important. Since the results show that low pH favors flavin hydroquinone and oxidized iron-sulfur center whereas high pH favors flavin semiquinone and reduced iron-sulfur center, the flavin reduction potential must increase relative to the iron-sulfur reduction potential with decreasing solvent pH.

The kinetic results presented here, particularly of the pH 6 $\rightarrow$ 8 and 8 $\rightarrow$ 6 experiments, indicate that the reequilibration of reducing equivalents within TMADH$_{2eq}$ is a kinetically complicated process. We have previously reported both static and kinetic difference spectra for the 10 $\rightarrow$ 7 and 7 $\rightarrow$ 10 pH-jumps (11). All of the spectral features observed in the pH 10 minus pH 7 static difference spectrum for TMADH$_{2eq}$ are quantitatively reproduced in the corresponding kinetic difference spectra obtained from both the 10 $\rightarrow$ 7 and 7 $\rightarrow$ 10 pH-jump reactions (although the two kinetic difference spectra are necessarily opposite in sign).6 Since the static and kinetic difference spectra agree so well in these experiments, we have concluded that there are no dead-time spectral changes in the course of these reactions. This contrasts with the results of the 6 $\rightarrow$ 8 pH-jump experiments reported here, particularly in the region around 365 nm, where there is a substantial discrepancy between the kinetic spectral changes observed kinetically (Figure 4) and those anticipated on the basis of the static spectra at these two pH values (Figure 2.1, dashed and dotted lines), presumably due to a significant dead-time spectral change in the kinetic experiments. The apparent discrepancy between the 7 $\rightarrow$ 10 and 6 $\rightarrow$ 8 experiments is resolved when one considers the difference in the state of
TMADH_{2eq} at pH 6 vs. pH 7; at pH 6 a substantial portion of the flavin present as the hydroquinone is protonated, whereas at pH 7 it exists predominantly as the anionic FMNH^-. If, during the low-to-high pH jump, intramolecular electron transfer must be preceded by the ionization of the N(1) position of flavin hydroquinone and this ionization exhibits a pK_a of 6 (both of these assertions are supported by recent studies of the reductive half-reaction; ref. 26), then approximately 90% of TMADH_{2eq} at pH 7 already exists in the form (anionic flavin hydroquinone and oxidized iron-sulfur center) observed at the conclusion of the 6 \rightarrow 8 pH-jump reaction. In other words, the absorbance increase at 365 nm which is lost in the dead time of the mixing apparatus in the 6 \rightarrow 8 pH-jump is undetectable as a dead-time spectral change in the 7 \rightarrow 10 pH-jump reaction since only 10% of the enzyme molecules undergo this process.

The multiphasic kinetic behavior and observation wavelength dependent rate constants observed in the 10 \rightarrow 8, 8 \rightarrow 10, and 6 \rightarrow 10 100\% D_2O pD-jump time courses, coupled with the mole fraction D_2O-dependent direction of absorbance change observed in the 8 \rightarrow 6 and 6 \rightarrow 8 100\% D_2O pD-jump time courses eliminate the possibility that the protonation/deprotonation and intramolecular electron transfer events occur concomitantly and indicate instead a reaction mechanism that entails discrete protonation/deprotonation and electron transfer steps. Scheme 1 represents the simplest overall reaction mechanism for prototropically controlled electron transfer within TMADH_{2eq} that is consistent with the known kinetic behavior of the enzyme.

Scheme 1

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The mechanism consists of discrete equilibria involving three prototropic equilibria and an intramolecular electron transfer step. Two of the three prototropic equilibria shown in scheme 1 involve ionizations of the flavin N(1) of hydroquinone and position N(5) of the semiquinone. The third prototropic equilibrium is between the protonated and unprotonated forms of an as-yet unidentified ionizable group whose ionization is required for formation of the spin-interacting state. The intramolecular electronic equilibrium involves electron transfer from anionic flavin hydroquinone to oxidized iron-sulfur center (to give neutral flavin semiquinone and reduced iron-sulfur center) and the reverse reaction.

In the course of a 10 → 6 pH-jump experiment, the unknown group and the N(5) position of anionic flavin semiquinone are first protonated, disrupting the interaction of the unpaired spins and forming the neutral flavin semiquinone. The order of protonation might be the reverse of that shown in Scheme 1, or protonation of these sites may occur simultaneously. Initial protonation of the unknown group followed by protonation of the anionic flavin semiquinone as drawn, however, is consistent with the observation of a lag phase in the 100% D₂O 10 → 8 time courses, and also with the results of reductive half-reaction studies with the slow substrate diethylmethylamine which indicate that formation of the spin-interacting state is kinetically distinct from intramolecular electron transfer, with little or no absorbance change associated with it (26). Subsequent to these two protonations, electron transfer from the iron-sulfur center to FMNH₂, forming the anionic hydroquinone which finally protonates (at sufficiently low pH) to give the neutral hydroquinone.

In principle, all of the active sites in a sample of TMADH₂eq will possess the neutral flavin hydroquinone at sufficiently low pH. At pH 6 (the lowest pH used in this and previous work and approximately the pKₐ of the hydroquinone) approximately 50% of the enzyme possesses the neutral form of the flavin hydroquinone (26). The remaining
50% of the active sites should consist of a mixture of [anionic flavin hydroquinone with oxidized iron-sulfur center], and [neutral flavin semiquinone and reduced iron-sulfur center] in a ratio of ~4:1. The visible absorbance spectrum of the enzyme species at the far left of Scheme 1 has been independently determined from reductive half-reaction studies of trimethylamine dehydrogenase since initial two-electron reduction of the FMN by substrate can be kinetically resolved from other processes taking place (7, 26); this is shown in Figure 2.1 (open circles).

Given that a simple two-exponential expression provides a satisfactory fit to the vast majority of the kinetic data presented here, kinetic simulations based on a mechanism with as many floating variables (eight rate constants) as depicted by Scheme 1 would certainly lead to meaningless fits to the data, and these were not attempted. As an alternative means to assess whether the proposed scheme is consistent with the data, an attempt has been made to account for the pH dependence of the observed rate constants for electron transfer within TMADH$_{2eq}$ in terms of Scheme 1. It is known that the observed rate constant for electron transfer within TMADH$_{2eq}$ in H$_2$O depends only on the final pH of the experiment (11). As shown in Figure 6, a plot of $k_{obs}$ vs. final pH plot is "U" shaped with a minimum near pH = 7.5, a reflection of the complex reaction mechanism required to describe prototropic control of intramolecular electron transfer in this enzyme. Since ionization of the unknown group with pK ~9.5 perturbs the electron distribution within partially reduced trimethylamine dehydrogenase only very slightly (as reflected in the EPR spectra observed at pH 8 and 10), consideration of only the FMN hydroquinone and semiquinone ionization states is to a good approximation sufficient to account for the observed pH dependence of $k_{obs}$. Omitting the far right equilibrium of Scheme 1 gives Scheme 2, in which the several microscopic rate constants for the interconversion of all intermediates are explicitly given:
Since the pH remains constant subsequent to the pH jump, the time rate of change for \([H^+]\) is zero and each of the protonation steps can be considered pseudo first-order. Scheme 2 is of the form \(A \rightleftharpoons B \rightleftharpoons C \rightleftharpoons D\). In situations in which the appearance of final product (either \(A\) or \(D\) depending on the direction of the pH-jump) follows first-order kinetics, as is observed in the present pH-jump reactions in \(H_2O\), an expression can be derived for the observed first order rate constant \((k_{obs})\) as a function of pH and the microscopic rate constants which describe the three equilibria (making a steady state approximation for the rate of change of intermediates \(B\), and \(C\); \(i.e.\) \(dB/dt = dC/dt = 0\)). Making these assumptions, the observed rate constant for intramolecular electron transfer as a function of proton concentration is given by the following equation:

\[
k_{obs} = \frac{(k_1 k_3 k_5 + k_1 k_3 k_6 + k_1 k_4 k_5 + k_2 k_4 k_6)}{(k_1 k_4 + k_1 k_5 + k_2 k_4 + k_2 k_5 + k_3 k_5 + k_1 k_3)}
\]  

(3)

The rate constants for the protonation of the flavin hydroquinone \((k_1)\) and semiquinone \((k_5)\) anions are equal to the corresponding bimolecular association rate constants multiplied by the proton concentration \((i.e. k_1 = k'_{hydroquinone} \times [H^+])\) and \(k_5 = k'_{semiquinone} \times [H^+]\). The rate constants for deprotonation \((k_2\) and \(k_6)\) are then defined in terms of the corresponding pseudo first-order protonation rate constants, the \(pK_a\) values of the appropriate flavin species, and the final pH value used in the experiment \((i.e. k_2 = k_1 \times (10^\alpha)\) with \(\alpha = pH_{final} - pK_{hydroquinone}\) and \(k_6 = k_5 \times (10^\beta)\) with \(\beta = pH_{final} -\)

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**pK**$_{\text{semiquinone}}$). The rate constants for electron transfer ($k_3$ and $k_4$) are considered pH independent parameters, reflecting the intrinsic rates of electron transfer from the reduced iron-sulfur center to the neutral semiquinone and from the anionic hydroquinone to the oxidized iron-sulfur center, respectively.

In fitting the $k_{\text{obs}}$ vs. final pH data to the above equation (Fig. 2.6, solid line), pK$_a$ values of 6.0 for the flavin hydroquinone and 8.5 for the semiquinone were used. The former value is consistent with recent reductive half-reaction studies of trimethylamine dehydrogenase (26). In the case of the pK$_a$ for the semiquinone, several attempts were made to fit the data using different values for the semiquinone pK$_a$ and it was found that a value of 8.5 yielded the best result. This value is within the range established by the optical/EPR spectral data (11) and the present kinetic results. The lower limit for the rate constants associated with the intramolecular electron transfer steps ($k_3$ and $k_4$) is 1000 s$^{-1}$; fits of the $k_{\text{obs}}$ vs. pH data to Equation 1 at different fixed values of $k_3$ and $k_4$ below 1000 s$^{-1}$ were unsatisfactory (data not shown). From the parameters giving the best fit to the pH dependence of $k_{\text{obs}}$, the bimolecular rate constants for protonation of the hydroquinone and semiquinone are calculated to be $2.6 \times 10^8$ M$^{-1}$ s$^{-1}$ and $1.0 \times 10^9$ M$^{-1}$ s$^{-1}$, respectively.

When considering the interrelationship between prototropic equilibria and intramolecular electron transfer in trimethylamine dehydrogenase, the protonation/deprotonation and electron transfer events may either occur concomitantly or as discrete chemical steps. Evidence for a discrete mechanism has been found in the kinetic and thermodynamic behavior of medium chain acyl-CoA dehydrogenase (29), while clear evidence for a concomitant mechanism has been found in the case of electron
Figure 2.6. *pH dependence of the observed rate constant for the TMADH$_{2eq}$ pH-jump.* The average of pH-jump (100% H$_2$O) $k_{obs}$ values obtained at 25 °C (taken from this work and ref. 11) plotted *versus* the experimental final pH (○). The *solid line* represents a fit of the $k_{obs}$ *vs.* pH data to Equation 3 using $pK_a$ values of 6.0 and 8.5 for the FMN hydroquinone and semiquinone respectively and rate constants for the electron transfer reactions ($k_3$ and $k_4$) = 1000 s$^{-1}$ (see text). The fitted values for the bimolecular rate constants for protonation of the FMN hydroquinone and semiquinone are $2.6 \times 10^8$ M$^{-1}$ s$^{-1}$ and $1.0 \times 10^9$ M$^{-1}$ s$^{-1}$ respectively.
transfer within xanthine oxidase (30). The present results indicate that electron transfer within trimethylamine dehydrogenase operates via a mechanism involving discrete ionization and electron transfer steps. The kinetic transients under many reaction conditions are complex (with either lags or multiple phases), the proton inventories indicate multiple ionizations involved in a $\text{pH } 6 \rightarrow 10$ jump or the reverse and the pH dependence of the reaction in $\text{H}_2\text{O}$ is distinctly non-linear. Xanthine oxidase, by contrast, exhibits well-behaved kinetics under all conditions, linear proton inventory plots indicative of the involvement of only a single proton in the electron transfer process and a linear dependence of $k_{\text{obs}}$ on pH. These results are not contradictory but are simply a reflection of different systems operating by different mechanisms.

Two factors in all likelihood combine to determine which type of mechanism is found in a given system. The first of these is the flavin redox couple participating in electron transfer; this is the quinone/semiquinone couple in xanthine oxidase and the semiquinone/hydroquinone couple in trimethylamine dehydrogenase. Since both semiquinone and hydroquinone, but not oxidized quinone, oxidation states of the flavin have ionizable protons, there is clearly greater likelihood for participation of multiple protons in the latter case than in the former. The second factor is the pK of the reduced form of the flavin that participates in the reaction. Thorpe and coworkers (29) point out that during the oxidation of a neutral semiquinone or hydroquinone, deprotonation to form the corresponding anionic species must precede electron transfer so as to avoid formation of an unfavorable cationic flavin species. Similarly, protonation of an anionic semiquinone should occur prior to reduction in order to avoid formation of an unfavorable dianionic hydroquinone. The protonation state of the flavin thus controls the kinetics as well as the thermodynamics of intramolecular electron transfer. For these reasons, a discrete mechanism for coupled proton/electron transfer might be expected for a given system in the absence of other considerations, as is observed in the cases of acyl-
CoA dehydrogenase and trimethylamine dehydrogenase. However, should the polypeptide preferentially destabilize the deprotonated form of the flavin by virtue of its hydrogen-bonding and other interactions with the isoalloxazine ring, then a discrete pathway might not necessarily reflect the lowest-energy path from the initial to the final states in the electron transfer process, in which case concomitant electron/proton transfer is preferred. This is apparently the case with xanthine oxidase.

The present results extend previous work indicating that electron transfer from the flavin hydroquinone of trimethylamine dehydrogenase to the iron-sulfur center of the enzyme is quite fast and is not intrinsically rate-limiting in catalysis. Further, evidence is found that formation of the spin-interacting state observed at the completion of the reaction of oxidized enzyme with substrate is governed by an ionizable group having a pK of approximately 9.5. Ionization of the flavin semiquinone to its anionic form also occurs in the course of formation of the spin-interacting state, with a pK of approximately 8.0. Given that only two redox-active centers are present in trimethylamine dehydrogenase, the enzyme might be considered a relatively simple system in which to examine electron transfer. Despite this apparent simplicity, the present results indicate a rather complicated reaction mechanism for the internal equilibration of reducing equivalents between the two centers that involves at least three discrete ionizations. These results combined with those obtained from other studies further indicate that two of these three ionizable groups are associated with the FMN coenzyme (in the semiquinone and hydroquinone oxidation state, respectively), while the third is most likely associated with an amino acid residue located at or near the active site (11, 26).
2.6 Footnotes

1. The abbreviations used are: TMADH, trimethylamine dehydrogenase; TMADH$_{2eq}$, trimethylamine dehydrogenase reduced to the level of 2 eq per subunit; FMN, flavin mononucleotide; 4Fe/4S, four iron-four sulfur center; Fe/S$_{ox}$, oxidized iron-sulfur center; Fe/S$_{red}$, reduced iron-sulfur center; EPR, electron paramagnetic resonance.

2. Since the two subunits of trimethylamine dehydrogenase appear to behave independently of each other, a single subunit will be referred to as one enzyme throughout this paper.

3. It is often possible to distinguish between the two forms of semiquinone in flavoproteins by EPR since the neutral form exhibits EPR line widths of ~19 gauss whereas the anionic form exhibits line widths of ~15 gauss (19-21). The flavin semiquinone of trimethylamine dehydrogenase exhibits line widths of 18.8 and 15.6 gauss at pH 6 and 10 respectively, and a pK$_a$ for the semiquinone of 8.0 has been estimated on the basis of the pH dependence of the linewidth (11). However, the signal intensity of the EPR signal at 150 K decreases significantly above pH 9 as the spin-interacting state accumulates, suggesting that the magnetic interaction between the unpaired electron spins of the flavin semiquinone and reduced iron-sulfur cluster (manifested directly in the EPR signal at 15 K) modulates the signal intensity of the EPR signal of the flavin radical observed at 150 K. The upshot is that the pK$_a$ of the flavin semiquinone cannot be sufficiently accurately determined from EPR line widths for the present purposes.

4. The rate constants presented with the kinetic time courses in Fig. 4A-M represent the average of at least six independent measurements for a given set of conditions. The errors associated with these values were determined as the standard deviation of the mean. In most cases, this uncertainty is approximately 20 - 25% of the value of the rate constant. However, the error (expressed as a percentage of the value of the rate constant) increases for kinetic time courses which exhibit either extremely large fitted rate constants (e.g. Fig. 2.4E-F) or a low signal-to-noise ratio due to a small absorbance change against a large background absorbance (e.g. 365 nm traces in Fig. 2.4I and 2.4L). In these cases, the uncertainty may increase to approximately 40% of the value of the associated observed rate constants (see also Table I in reference 11).

5. The difference between the values of the observed rate constants obtained at 410 nm ($k_{obs} = 200$ s$^{-1}$) and 520 nm ($k_{obs} = 250$ s$^{-1}$) for the $8 \rightarrow 6$ pH-jump reaction is considered significant as it is just beyond the limit of experimental error associated with these measurements.

6. See Figure 7 in reference 11.
7. Using the slow substrate diethylmethylamine, it has been shown that there is a small spectral change associated with formation of the spin-interacting state (26). This spectral change is identical, however, to that associated with electron transfer from the flavin hydroquinone to the iron-sulfur center of trimethylamine dehydrogenase. It has been concluded that the spectral change associated with formation of the spin-interacting state is due to a small (~10%) reequilibration of reducing equivalents between the two redox-active centers, favoring further iron-sulfur reduction, upon formation of the spin-interacting state rather than a distinctive absorbance intrinsic to the state itself.

8. We emphasize that this analysis is valid only for the results obtained in H$_2$O, where the observed transients are well-represented as single-exponential processes. In addition, the data plotted in Figure 6 are exclusively from experiments in which the initial pH was either 6 or 10, although the results of experiments jumping either to or from the intermediate pH 8 are not inconsistent with the proposed mechanism. It may seem paradoxical that the pH6 $\rightarrow$ 8 kinetics (k$_{obs}$ = 400 s$^{-1}$) are slower than for the pH 6 $\rightarrow$ 10 experiment (k$_{obs}$ = 1000 s$^{-1}$), but it must be remembered that the observed kinetics are intrinsically dependent on the hydrogen ion concentration. In the pH 6 $\rightarrow$ 10 experiment, there is no reason why the approach to the final state achieved at pH 8 cannot be faster than in the pH 6 $\rightarrow$ 8 experiment, as is in fact empirically observed and also predicted by the model. For the reverse change in pH, it is difficult to rationalize a priori why the pH 8 $\rightarrow$ 6 kinetics are slower than the pH 10 $\rightarrow$ 6 kinetics at two of the three observation wavelengths followed here. Given the pronounced wavelength dependence of the observed rate constant in these experiments and the evidence presented for dead-time spectral changes, it is in any case not appropriate to include these data in the analysis of the pH dependence of k$_{obs}$. We cannot exclude the possibility of a more complicated, perhaps branched mechanism, to quantitatively account for the observed pH-jump kinetics to and from pH 8, but note that the results of these experiments are at least qualitatively consistent with the mechanism given in Schemes 1 and 2.
LIST OF REFERENCES


CHAPTER 3

THE REACTION OF TRIMETHYLAMINE DEHYDROGENASE WITH ELECTRON TRANSFERRING FLAVOPROTEIN

3.1 Abstract

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 ($k_{lim}$) 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 one-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 two-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.
3.2 Introduction

Trimethylamine dehydrogenase (TMADH\(^1\); 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-cysteiny1 FMN coenzyme and a 4Fe/4S (ferredoxin-type) iron-sulfur center (1-4). TMADH also possesses one equivalent of tightly bound ADP per 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 ab dimer of molecular weight of 77,000 Da and contains one mol of FAD and AMP per mol of protein; the role of AMP is unclear.

Complete reduction of TMADH requires three electrons per subunit, two for full reduction of the FMN, and a third for reduction of the iron-sulfur center. When TMADH is reduced to the level of two electrons per subunit, there are two possible distributions of reducing equivalents: 1) fully reduced FMN with oxidized iron-sulfur and 2) flavin semiquinone with 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 (TMAC) (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\textsubscript{2eq*} is mixed with ETF\textsubscript{ox}, the EPR signal arising from the spin-interacting state is lost within a few ms 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\textsubscript{2eq*} to ETF\textsubscript{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\textsubscript{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.

3.3 Materials and Methods

Enzyme Purification and Materials - 

*Methylophilus methylotrophus* W\textsubscript{3A1} 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 mM\textsuperscript{-1}cm\textsuperscript{-1} (3). Enzyme assay was performed as described by McIntire (12). ETF from *M. methylotrophus* W\textsubscript{3A1} 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 mM\(^{-1}\)cm\(^{-1}\) (7). Phenylhydrazine-inactivated TMADH was prepared as described by Kasprzak et al. (13, 14). The concentration of TMADH thus inactivated was determined using an extinction coefficient of 14.8 mM\(^{-1}\)cm\(^{-1}\) at 442 nm (13, 14). Phosphate buffer was obtained from Sigma, and boric acid from Jenneil Chemical Co.. Sodium dithionite was obtained from Virginia Chemicals and phenyl hydrazine from Eastman Kodak. Ferricenium 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 TiCl\(_3\) in 2.0 M hydrochloric acid (purchased from Aldrich) to an appropriate volume of 0.1M 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 photoreduction), and made anaerobic by alternately evacuating and flushing with O\(_2\)-free argon. Solutions of reduced TMADH were prepared by being made anaerobic as above, then titrated with either titanium citrate or sodium dithionite. 1.0 ml samples 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 O\(_2\)-free argon. 1.0 ml of anaerobic oxidized ETF 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 five time 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 non-linear least squares Levenberg-Marquardt algorithm (18), using the expression \( \Delta A(t) = \Sigma \Delta A_n \exp(-k_n t) \) (with the floating variables \( \Delta A_n \) and \( k_n \) representing the absorbance change and observed rate constant, respectively for the nth kinetic phase).

3.4 Results

The Spectral Change Associated with Reaction of Reduced TMADH with Oxidized ETF - The absorption spectra for oxidized, two- and three-electron reduced TMADH are shown in Figure 3.1A, and the spectra for oxidized and semiquinone forms of ETF are shown in Figure 3.1B. To determine the absorbance change associated with electron transfer from TMADH to ETF, the following experiments were performed. 1.0 ml of 8.2 \( \mu \)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 \( \mu \)M ETF\textsubscript{ox} 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 absorption increase in the 300-410 nm and 462-600 nm ranges and a decrease in the 410-462 nm range (Fig. 3.1C). To demonstrate that the spectral change is quantitatively consistent with the reduction of ETF\textsubscript{ox} and the reoxidation of TMADH\textsubscript{3eq} in a 3:1
**Figure 3.1.** Panel A: TMADH optical spectra. The spectra shown are for oxidized enzyme (solid line), two-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 per 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 μM) was mixed anaerobically with 50 μM of oxidized ETF in 50 mM phosphate buffer, pH 7.0 in a split cell (see Material 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 two-electron-reduced TMADH with oxidized ETF. TMADH (14 mM) reduced with titanium citrate to the level of 2eq per subunit was mixed anaerobically with 42 μM 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 TMADH$_{3eq}$ with ETF$_{ox}$. The theoretical difference spectrum (dotted line) was obtained by adding the difference spectrum for reoxidation of 4.1 μM TMADH$_{3eq}$ (Panel A) to the difference spectrum for reduction of 12.3 μM ETF$_{ox}$ (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.
Fig. 3.1
Figure 3.1 (continued)
Figure 3.1 (continued)
Figure 3.1 (continued)
Figure 3.1 (continued)
stoichiometry, the difference spectra for reoxidation of TMADH$_{3\text{eq}}$ and reduction of 3 equivalents of ETF$_{\text{ox}}$ were generated from the spectra of Figure 3.1 (A and B) and added. The difference spectrum thus obtained (Fig. 3.1E, dotted line) is very similar to the experimental difference spectrum (Fig. 3.1E, solid line), demonstrating that one equivalent of TMADH$_{3\text{eq}}$ reduced three equivalents of ETF$_{\text{ox}}$. 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 ETF$_{\text{ox}}$ 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 1.0 ml of 14 $\mu$M TMADH$_{2\text{eq}}$ in the non-spin-interacting state (generated by titration with Ti$^{III}$citrate to the level of two reducing equivalents per subunit at pH 7.0) is mixed with 1.0 ml of 42 $\mu$M ETF$_{\text{ox}}$, the spectral change shown in Fig. 3.1D is observed and found to be quantitatively consistent with the reduction of ETF$_{\text{ox}}$ and the reoxidation of TMADH$_{2\text{eq}}$ in the ratio of 2:1. When TMADH$_{2\text{eq}}$ in the spin-interacting state (generated by reduction with one equivalent of trimethylamine in the presence of 3 mM tetramethyl ammonium chloride) is mixed anaerobically with ETF$_{\text{ox}}$ at pH 7.0 in a split cell, the spectral change is again consistent with the reduction of ETF$_{\text{ox}}$ and the reoxidation of the spin interacting state of TMADH$_{2\text{eq}}$ 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 Ferricenium-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. 3.2A) is due entirely to reduction of the enzyme iron-sulfur center. When reduced, phenylhydrazine-inactivated enzyme is mixed anaerobically with ETF$_{ox}$ in a split cell experiment of the type described above, the absorbance change shown in Figure 3.2B 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 ETF$_{ox}$ and the reoxidation of the reduced, inactivated TMADH in a stoichiometry of 1:1 (Fig. 3.2C). 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 ETF$_{ox}$ 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 4Fe/4S 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. 3.3).

Treatment of TMADH with 3 mM ferricenium hexafluorophosphate at pH 10 for 4 hours at room temperature has been found empirically to give an iron-sulfur center that is EPR-active (see Chapter 5). The EPR spectrum of the enzyme
Figure 3.2. Panel A: Phenylhydrazine-inactivated TMADH optical spectra. The spectra shown are for oxidized enzyme (solid line) and dithionite reduced enzyme (dotted line) in 50 mM phosphate buffer, pH 7.0. Panel B: Optical spectra observed before and after mixing of reduced phenylhydrazine-inactivated TMADH with oxidized ETF. 1 ml of 12 μM phenylhydrazine inactivated TMADH in 50 mM phosphate buffer, pH 7.0 was reduced with dithionite and then placed in one side of an anaerobic split cell. 1 ml of 34 μM anaerobic oxidized ETF in 50 mM phosphate buffer, pH 7.0 was placed in the other side of the split cell. The solid line is the spectrum recorded before mixing of phenylhydrazine-inactivated TMADH with oxidized ETF and the dotted line is the spectrum recorded after mixing. Panel C: Theoretical and experimental difference spectra for the reaction of reduced phenylhydrazine-inactivated TMADH with ETF$_{ox}$. The theoretical difference spectrum (dotted line) was obtained by adding the difference spectrum for reoxidation of 6 μM reduced phenylhydrazine-inactivated TMADH (Panel A) to the difference spectrum for reduction of 6 μM ETF$_{ox}$. The experimental difference spectrum (solid line) was obtained by subtracting the solid line in Panel B from the dotted line in Panel B.
Fig. 3.2

Absorbance

Wavelength (nm)
Figure 3.2 (continued)
Figure 3.2 (continued)
Figure 3.3. Deconvolution of the difference spectrum for oxidized and fully reduced TMADH. The difference spectrum for oxidized and reduced 4Fe/4S center (solid line) is obtained by subtracting the spectrum for reduced phenylhydrazine-inactivated TMADH from that for oxidized inactivated TMADH. The difference spectrum for oxidized and reduced TMADH (dashed line) is obtained by subtracting the spectrum for fully reduced TMADH from that for oxidized TMADH. The difference spectrum for the enzyme FMN and FMNH$_2$ (dotted line) is obtained by subtracting the difference spectrum for 4Fe/4S center from that for TMADH.
thus generated is found to superficially resemble that given by the oxidized form of various 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. The Mössbauer and MCD results show that the modified iron-sulfur center is neither a 3Fe cluster, nor a HiPIP 4Fe-4S center. No attempt has been made to further identify the modified iron-sulfur center in ferricenium-treated TMADH since the Mössbauer and MCD data are complicated. 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 mins does not reduce the intensity of the new EPR signal.² 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 ETF₀ₓ, 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 ETF₀ₓ at pH 7.0 exhibits two kinetic phases (Fig. 3.4A). The fast phase of the reaction accounts for 80% of the total spectral change and the observed rate constant is ETF concentration dependent, and under pseudo first-order conditions
Figure 3.4. Time courses observed for the reactions of reduced phenylhydrazine-inactivated TMADH and 3 electron reduced TMADH with oxidized ETF. Absorbance changes observed at 370 nm (closed triangles) and 440 nm (closed circles) after mixing in a stopped-flow apparatus are plotted versus time. The reaction conditions are 50 mM potassium phosphate, pH 7.0, 25 °C. The symbols represent the data points, and the solid lines represent fits of the data to exponentials of the form \( \Delta A(t) = \sum A_n \exp(-k_n t) \) where \( A_n \) and \( k_n \) represent the absorbance change and observed rate constant exhibited by the \( n \)th kinetic phase, respectively. Panel A, reaction of dithionite reduced phenylhydrazine-inactivated TMADH with ETF\(_{ox}\). The concentrations after mixing are: [inactivated TMADH]=3 \( \mu \)M, [ETF]=58 \( \mu \)M. Data are fitted to the sum of two exponentials: \( k_{fast} = 141 \text{ s}^{-1} \), \( k_{slow} = 3 \text{ s}^{-1} \). The observed rate constants for each kinetic phase are independent of observation wavelength. Panel B, reaction of TMADH\(_{3eq}\) with ETF\(_{ox}\). After mixing, [TMADH]=1 \( \mu \)M, [ETF]=45 \( \mu \)M. Only every tenth point is shown to allow visualization of the fitted curves. Data at 370 nm are fitted to the sum of two exponentials: \( k_{fast} = 138 \text{ s}^{-1} \) and \( k_{slow} = 12 \text{ s}^{-1} \). Data at 440 nm are fitted to a single exponential: \( k = 12 \text{ s}^{-1} \).
Fig. 3.4

\[ \Delta A \]

\[ T \text{ (sec)} \]

A

B

\[ \Delta A \]

\[ T \text{ (sec)} \]

88
this phase is first-order. A double reciprocal plot (21) of $k_{\text{obs}}$ versus ETF concentration is linear (Fig. 3.5, open squares), giving values of $k_{\text{lim}}$ and $K_d$ of 173 s$^{-1}$ and 16 μM, respectively. The wavelength dependence of DA for the fast phase is consistent with it being due to electron transfer from the iron-sulfur center of inactivated TMADH to ETF and this phase thus reflects the intrinsic kinetic parameters for the reaction of the fully reduced iron-sulfur center with oxidized ETF. The slow phase of the reaction, with an [ETF]-independent rate constant of $\sim$ 3 s$^{-1}$, and given its relatively minor contribution (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. 3.6), indicating that there is no absorbance loss in the dead time of the stopped-flow apparatus. To investigate the ionic 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 ox in 50 mM KP$_4$, 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 s$^{-1}$ and $K_d$=16 μM (table 3.1), not much different from the values obtained in the absence of KCl. The fact that ionic 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_{red}ETFOX complex.

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 ox at 5, 15, 25, and 35 °C. The observed rate constants were temperature dependent and the Arrhenius plot is linear (Fig. 3.7), giving activation energy of 12.8 kcal/mol.
**Figure 3.5.** Double-reciprocal plot of the observed rate constants versus ETF concentration. All reactions were measured in a stopped-flow apparatus at 370 nm and 25 °C. The buffer was 50 mM potassium phosphate, pH 7.0. The open circles represent the data for the fast phase of the reaction of 3 electron reduced TMADH with ETF. The enzyme concentration after mixing was 1.0 mM. The fit of the data gives $k_{\text{lim}}=172 \text{ s}^{-1}$ and $K_d=10 \mu\text{M}$. The open squares represent the data for the reaction of phenylhydrazine-inactivated TMADH with ETF. The concentration of inactivated TMADH after mixing was 3.0 μM. The fit of the data gives $k_{\text{lim}}=173 \text{ s}^{-1}$ and $K_d=16 \mu\text{M}$. The filled circles represent the data for the fast phase of TMADH$_{2\text{eq}}$ in the spin-interacting state with ETF. The enzyme concentration after mixing was 2.3 μM. The fit of the data gives $k_{\text{lim}}=149 \text{ s}^{-1}$ and $K_d=39 \mu\text{M}$. The filled squares represent the data for the fast phase of the reaction of TMADH$_{2\text{eq}}$ in non-spin-interacting state with ETF. The concentration of TMADH after mixing was 2.1 μM. The fit of the data gives $k_{\text{lim}}=157 \text{ s}^{-1}$ and $K_d=24 \mu\text{M}$.
Table 3.1 The intrinsic limiting rate constants (k_{lim}) and dissociation constants (K_d) for the fast phases of the reactions of various forms of TMADH with ETF

<table>
<thead>
<tr>
<th>Reactions</th>
<th>k_{lim}</th>
<th>K_d</th>
</tr>
</thead>
<tbody>
<tr>
<td>reduced, phenylhydrazine-inactivated TMADH + ETF</td>
<td>173</td>
<td>16</td>
</tr>
<tr>
<td>reduced, phenylhydrazine-inactivated TMADH + ETF 0.2 M KCl</td>
<td>125</td>
<td>16</td>
</tr>
<tr>
<td>fully reduced TMADH + ETF</td>
<td>172</td>
<td>10</td>
</tr>
<tr>
<td>TMADH2eq (non-spin-interacting state) + ETF</td>
<td>157</td>
<td>24</td>
</tr>
<tr>
<td>TMADH2eq (spin-interacting state) + ETF</td>
<td>149</td>
<td>39</td>
</tr>
</tbody>
</table>
Figure 3.6. *Static and kinetic difference spectra.* Panel A, the solid line is the static difference spectrum calculated from the spectrum taken after mixing of TMADH$_{2eq}$ (14 μM) with ETF$_{ox}$ (42 μM) at pH 7.0 minus the spectrum recorded before mixing (see Fig. 3.1D). The close 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 phenyl hydrazine 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 phenyl hydrazine 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.
Fig. 3.6

![Graph showing absorbance vs. wavelength (nm)](image)

Absorbance

Wavelength (nm)
Figure 3.6 (continued)
Figure 3.7. Temperature dependence of the observed rate ($k_{obs}$) for the reaction of reduced phenyl hydrazine inactivated TMADH with oxidized ETF. Dithionite reduced phenyl hydrazine inactivated TMADH (final concentration, 4.3 μM) was mixed with oxidized ETF (final concentration, 44 μM) in a stopped-flow apparatus. The buffer used was 50 mM potassium phosphate, pH 7.0. The values of $k_{obs}$ used in the Arrhenius plot were obtained from fits of kinetic transients to a single exponential expression and represent the average of at least five independent measurements at a given temperature.
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 (approximately 67%) appears to be kinetically significant. Fig. 3.4B shows kinetic transients obtained on mixing TMADH$_{3eq}$ with excess ETF$_{ox}$ at pH 7.0 in a stopped-flow spectrophotometer. The transient observed at 370 nm consists of both phases, while that observed at 440 nm contains only the slow phase. The observed rate constants for each kinetic phase at a given set of reaction conditions are found to be independent of observation wavelength over the range of 300 - 600 nm. At 370 nm, the fast phase accounts for about one third 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. 3.5, open circles) and the fit of the data gives $k_{lim}=172$ s$^{-1}$ and $K_d=10$ μM, in good agreement with the results using phenylhydrazine-inactivated enzyme. The observed rate constant for the slow phase is also ETF concentration dependent and the double reciprocal plot of $k_{obs}$ versus ETF concentration is linear (Fig. 3.8), giving $k_{lim}=16$ s$^{-1}$ and $K_d=9.9$ μM. The overall kinetics are consistent with the rapid removal of the first reducing equivalents from fully reduced TMADH, followed by the much slower removal of the second and third equivalents from the two-electron reduced enzyme thus generated (owing to an unfavorable distribution of the reducing equivalents in TMADH$_{2eq}$ and TMADH$_{1eq}$, see discussion).

It is of interest to determine whether the rate constant 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. 3.8 Double reciprocal plot of the observed rate constants for the slow phase versus ETF concentration. The symbols represent the observed rate constants for the slow phase of the reaction of 3-electron reduced TMADH with ETF. The reaction conditions are: [TMADH]=1.0 μM, 370 nm, 25 °C, 50 mM potassium phosphate, pH 7.0.
(Fig. 3.5, filled squares) and the fit of the data gives $k_{\text{lim}}=157 \text{ s}^{-1}$ and $K_d=24 \mu\text{M}$. The observed rate constant for the slow phase is approximately $10 \text{ s}^{-1}$ and is independent of [ETF] under pseudo first-order conditions. In this experiment, approximately 40% of the TMADH$_{2\text{eq}}$ 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$_{2\text{eq}}$ in the spin-interacting state (generated by reduction of the enzyme with one 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 $k_{\text{lim}}$ and $K_d$ of $149 \text{ s}^{-1}$ and $39 \mu\text{M}$, respectively (Fig. 3.5, filled circles). The rate constant for the slow phase, which accounts for approximately 30% of the total absorbance change, is about $4 \text{ s}^{-1}$ and independent of ETF concentration.

3.5 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 ferricenium hexafluorophosphate at high pH, oxidizing the iron-sulfur center to a paramagnetic but redox-inert state, the FMN can be reduced by 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 reductive half-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 $k_{\text{lim}}$ and $K_d (16 \mu\text{M})$ are insensitive to high salt concentration indicates that electrostatic forces are not involved in the formation and decay of $E_{\text{red-ETF}}$ complex. The good agreement between both $k_{\text{lim}}$ and $K_d$ for the reoxidation of reduced, phenylhydrazine-inactivated TMADH with ETF$_{\text{ox}}$ and the fast phase of the reoxidation of 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$_{3\text{eq}}$ with ETF accounts for one third of the total absorbance change and $k_{\text{lim}}$ 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$_{2\text{eq}}$. 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$_{2\text{eq}}$, 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$_{3\text{eq}}$ and TMADH$_{2\text{eq}}$ (16 s$^{-1}$ and 10 s$^{-1}$, respectively). The small rate constants for both slow phases owe to the unfavorable distribution of the reducing equivalents in TMADH$_{2\text{eq}}$ and TMADH$_{1\text{eq}}$. The distribution of the reducing
equivalents favors the enzyme form with FMNH\textsubscript{2} and oxidized 4Fe/4S in TMADH\textsubscript{2eq} and with FMNH\textsuperscript{-} and oxidized 4Fe/4S in TMADH\textsubscript{1eq}. The reoxidation of fully reduced TMADH by ETF can thus be summarized as shown in Scheme 1:

![Scheme 1](image)

The fast phases of the reactions of ETF with TMADH\textsubscript{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\textsubscript{2eq} in non-spin-interacting state and all the 4Fe/4S center in TMADH\textsubscript{2eq} in spin-interacting state are reduced. \( k_{\text{lim}} \) for the fast phases of the two reactions are within experimental error (15-20%, ref. 23) of the value for the reaction of phenylhydrazine-inactivated TMADH with ETF. Thus, both fast phases of the reaction of ETF with TMADH\textsubscript{2eq} in non-spin-interacting state and in spin-interacting state represent the electron transfer from the reduced 4Fe/4S center in TMADH\textsubscript{2eq} to ETF and the rate of the electron transfer is independent of the reduction state of the FMN. \( k_{\text{lim}} \) (149 s\textsuperscript{-1}) for the fast phase of the reaction of TMADH\textsubscript{2eq} in the spin-interacting state with ETF is identical, within experimental error, to that for the fast phase of the reaction of TMADH\textsubscript{2eq} in non-spin-interacting state with ETF (157 s\textsuperscript{-1}). This indicates that formation of the spin-interacting
state does not significantly slow down the electron transfer from the 4Fe/4S center to ETF and that binding of tetramethylammonium chloride has little effect on the rate of electron transfer from the iron-sulfur center of TMADH to ETF. As in the absence of tetramethylammonium chloride, the slow phase represents the oxidation of TMADH\textsubscript{1eq}, in which the sole reducing equivalent resides primarily on the FMN center. The somewhat slower rate constant for the slow phase of the reaction in the presence of tetramethylammonium chloride (4 s\textsuperscript{-1} versus 10 s\textsuperscript{-1} in its absence) is consistent with the observation that binding of tetramethylammonium chloride raises the FMN/FMNH\textsuperscript{-} half-potential (19), thereby further shifting the oxidation-reduction equilibrium within TMADH\textsubscript{1eq} even further toward flavin reduction and iron-sulfur oxidation and slowing the rate of reaction with ETF.

Our kinetic results concerning the reaction of the various reduced forms of TMADH with ETF can be incorporated into a comprehensive kinetic mechanism for the turnover of TMADH with trimethylamine and ETF. Because of the unusual situation where reducing equivalents are introduced into the enzyme in pairs (at the FMN) and removed one at a time (at the iron-sulfur center), coupled with the ability of the enzyme to take up a total of three equivalents, the general mechanism shown in Scheme 2 must be considered. Two alternate paths exist in which the enzyme alternates between (1) oxidized and two-electron reduced forms (the "0/2 Cycle" shown on the left of Scheme 2, prevailing at low concentrations of trimethylamine) or (2) between one- and three-electron reduced forms (the "1/3 Cycle" shown on the right, prevailing at high concentrations of trimethylamine). The cycle in which the enzyme operates depends on the fate of TMADH\textsubscript{2eq} formed after reaction of oxidized enzyme with one equivalent of trimethylamine. This species can react either with ETF to give oxidized enzyme, or with substrate to give the spin-interacting state, which subsequently leads to formation of fully reduced enzyme. Ultimately which branch is favored under a given set of experimental...
conditions depends on the relative concentrations of ETF and TMA and their relative rates of reaction with enzyme.

The reductive half-reaction of TMADH has been studied by using both substrate trimethylamine and nonphysiological substrate, diethylmethylamine (9-11, 23, 24). Initial reduction of the enzyme by trimethylamine occurs at the flavin site and is very rapid ($t_{1/2} < 2 \text{ ms}$, $[\text{TMA}]=500 \text{ mM}$). Following this initial rapid reduction, two slower kinetic phases ($t_{1/2}$ are approximately 80 ms and 200 ms, respectively) are observed. The rate constant for the slowest phase is approximately equal to $k_{\text{cat}}$ (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 two-electron reduced enzyme is the rate-limiting step (23). Intramolecular electron transfer within
TMADH\textsubscript{2eq} has been studied using a pH jump technique and intramolecular equilibration of reducing equivalents is fast ($k_{\text{obs}} \gtrapprox 200 \text{ s}^{-1}$) (25).
3.6 Footnotes

1. The abbreviations used are: ETF, electron transferring flavoprotein; ETF$_{ox}$, oxidized electron transferring protein; ETF$_{sq}$, electron transferring flavoprotein semiquinone; TMADH, trimethylamine dehydrogenase; TMADH$_{2eq}$, trimethylamine dehydrogenase reduced to the level of 2 eq per subunit; TMADH$_{2eq}^*$, spin-interacting state of TMADH; TMADH$_{3eq}$, fully reduced trimethylamine dehydrogenase; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; FMNH$_2$, flavin mononucleotide hydroquinone; FMN$_{sq}$, flavin mononucleotide semiquinone; 4Fe/4S$_{ox}$, oxidized four iron-four sulfur center; 4Fe/4S$_{red}$, reduced four iron-four sulfur center; EPR, electron paramagnetic resonance; TMAC, tetramethylammonium chloride.

2. 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.

3. 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 one electron per 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.
LIST OF REFERENCES


CHAP﻿TER 4

THE REACTION OF THE C30A MUTANT OF TRIMETHYLAMINE DEHYDROGENASE WITH DIETHYLMETHYLAMINE

4.1 Abstract

The role played by the 6-S-cysteiny1 FMN bond of trimethylamine dehydrogenase in the reductive half-reaction of the enzyme has been studied by following the reaction of the slow substrate diethylmethylamine with a C30A mutant of the enzyme lacking the covalent flavin attachment to the polypeptide. Removal of the 6-S-cysteiny1 FMN bond diminishes the limiting rate for the first of the three observed kinetic phases of the reaction by a factor of six, but has no effect on the rate constants for the two subsequent kinetic phases. The flavin in the C30A enzyme recovered from the reaction of C30A enzyme with excess substrate is found to have been converted to the 6-hydroxy derivative, rendering the enzyme inactive. The noncovalently bound FMN of the C30A mutant enzyme is also converted to 6-hydroxyFMN and rendered inactive upon reduction with excess trimethylamine, but not by reduction with dithionite, even at high pH or in the presence of the effector tetramethylammonium chloride. These results demonstrate that one significant role of the 6-S-cysteiny1 FMN bond is to prevent the inactivation of the enzyme during catalysis. A reaction mechanism is proposed whereby OH\textsuperscript{-} attacks
C(6) of a flavin-substrate covalent adduct in the course of steady-state turnover to form 6-hydroxyFMN.
4.2 Introduction

Trimethylamine dehydrogenase (EC 1.5.99.7) purified from the restricted facultative methylotroph *Methylophilus methylotrophus* is composed of two identical 83,000 Da subunits. Each subunit contains one equivalent of FMN and a ferredoxin-type 4Fe-4S cluster, along with one equivalent of ADP of unknown function (1-6). The enzyme catalyzes the oxidative demethylation of trimethylamine according to the following stoichiometry:

\[
(CH_3)_3N + H_2O \rightleftharpoons (CH_3)_2NH + HCHO + 2H^+ + 2e^-
\]

During catalysis, the FMN of trimethylamine dehydrogenase takes up two reducing equivalents from substrate and subsequently transfers them one at a time via its 4Fe-4S center to the physiological oxidant for the reaction, electron transferring flavoprotein (7-12). The crystal structure of trimethylamine dehydrogenase has been determined at 2.4-Å resolution (13, 14) and it is found that each subunit is folded into three discrete domains: a large N-terminal domain and two smaller central and C-terminal domains. The large N-terminal domain is folded into a parallel α8β8 barrel and contains the FMN and iron-sulfur center. The central domain contains a 5-stranded parallel β-sheet flanked by α-helices on each side; the C-terminal domain also contains a 5-stranded parallel β-sheet, but is flanked by α-helices on one side and an antiparallel 3-stranded β-sheet on the other. The FMN is covalently linked to Cys-30 of the large N-terminal domain as the 6-cysteinyl derivative while the iron-sulfur center is located close to the central domain. The 8α-methyl of FMN and the closest cysteinyl sulfur atom of iron-sulfur center are only 4 Å apart (13). The two groups are sufficiently close that in the presence of excess substrate or tetramethylammonium chloride (a known inhibitor of the enzyme, ref. 15) or at high pH, the unpaired electron spins of the flavin semiquinone and reduced iron-

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sulfur center of two-electron reduced enzyme interact strongly to give a triplet-like state which exhibits a complex EPR signal at \( g=2 \) and an intense half-field \( (g=4) \) signal (11).

The reaction of trimethylamine dehydrogenase with the slow substrate diethylmethylamine has been investigated recently, and the reaction is found to exhibit three well-resolved kinetic phases (9). During the fast kinetic phase, the absorbance at 450 nm attributable to oxidized FMN is lost in a process that has been interpreted as giving rise to a covalent flavin-substrate adduct. The intermediate kinetic phase represents the breakdown of this covalent adduct and subsequent rapid intramolecular electron transfer of one electron from the flavin hydroquinone thus formed to the iron-sulfur center. The slow kinetic phase has been interpreted as representing the dissociation of product followed by the binding of a second substrate molecule to the 2-electron reduced enzyme, a process that coincides with the formation of the spin-interacting state (9). The oxidative half-reaction has also been studied using various forms of trimethylamine dehydrogenase and electron transferring flavoprotein (10). It has been demonstrated that electrons are transferred from trimethylamine dehydrogenase to electron transferring flavoprotein via the iron-sulfur center of the former with a limiting rate constant of 172 s\(^{-1}\) (at pH 7.0 and 25 °C); this step is thus not rate-limiting in catalysis (10).

As mentioned above, the FMN in trimethylamine dehydrogenase is covalently attached to Cys-30 via the C(6) position of the isoalloxazine ring by a 6-S-cysteinyl FMN bond (1, 16,17). When Cys-30 of the enzyme is replaced with alanine by site-directed mutagenesis, the C30A mutant enzyme contains the full complement of 4Fe-4S and ADP, but only about 30% of the full complement of (now noncovalently bound) FMN (18, 19). To the extent that the C30A mutant possesses flavin, however, the mutant enzyme is catalytically active, albeit somewhat compromised. Steady-state kinetic experiments show that the removal of the 6-S-cysteinyl FMN bond increases the apparent \( K_m \) for
trimethylamine by a factor of one hundred and decreases the apparent $k_{cat}$ by a factor of two (18). The steady-state kinetic analysis, however, does not provide direct information about the role of the 6-S-cysteinyl FMN bond in accelerating any of the specific steps of the reductive half-reaction. In the present study, we have investigated the reaction of C30A mutant trimethylamine dehydrogenase with diethylmethylamine in order to examine in greater detail the role of the covalent flavin linkage in catalysis.

4.3 Materials and Methods

Enzyme Purification and Materials - E. coli strain JM109 transformed with the plasmid pSV2tmdvegC30A was grown and C30A mutant trimethylamine dehydrogenase was purified as described by Scrutton et al. (18). Enzyme concentrations were determined using the extinction coefficient of 197.1 mM$^{-1}$cm$^{-1}$ at 280 nm which is calculated from native trimethylamine dehydrogenase. Diethylmethylamine was purchased from Aldrich and potassium phosphate, tetrasodium pyrophosphate, trimethylamine chloride, FMN, and riboflavin were obtained from Sigma; sodium dithionite was obtained from Virginia Chemicals. Ferricenium hexafluorophosphate was prepared as described by Lehman et al. (20, 21).

Enzyme Assays - Enzyme assays were performed in 0.1 M potassium phosphate buffer, pH 7.5, 25 °C, which contained 200 µM diethylmethylamine and 200 µM ferricenium hexafluorophosphate in a total volume of 1.0 ml. Reactions were initiated by the addition of 10 µl of enzyme solution and the decrease in absorbance at 300 nm was recorded for 10 min using a Hewlett-Packard 8452A single beam diode array spectrophotometer; assay without enzyme was used as a control. The amount of ferricenium hexafluorophosphate reduced was calculated using an extinction coefficient of 4300 M$^{-1}$cm$^{-1}$ at 300 nm (20) and enzyme turnover was calculated by dividing the
amount of ferricenium hexafluorophosphate reduced per minute by two to give a value reflecting turnover with respect to oxidative demethylation of the amine substrate.

**Kinetic Experiments** - Pre steady-state studies were carried out using a Kinetic Instruments Inc. stopped-flow apparatus equipped with an On Line Instruments System (OLIS) model 3920Z data collection system. Anaerobic solutions of oxidized C30A mutant trimethylamine dehydrogenase were prepared by alternately evacuating and flushing with O₂-free argon in a tonometer equipped with a side arm cuvette and a three-way stopcock valve possessing a male Luer connector. Anaerobic solutions of the volatile diethylmethylamine were prepared by bubbling buffer solution with O₂-free argon for 30 mins in a 10-ml volume glass syringe and then injecting an appropriate volume of diethylmethylamine to give a final concentration of 20 mM. The glass syringe was equipped with a three-way valve to allow serial dilution of the diethylmethylamine stock solution thus prepared. The final concentrations of diethylmethylamine after mixing in the present studies were between 1.25 mM and 10 mM, sufficient to ensure pseudo first-order conditions in the stopped-flow experiments. Kinetic transients were obtained as transmittance voltage as a function of time and converted to absorbance change using OLIS software. Kinetic transients thus obtained were fitted to the sum of exponentials \( \Delta A(t) = \sum \Delta A_n \exp(-k_n t) \), with \( \Delta A_n \) and \( k_n \) representing the absorbance change and observed rate constant, respectively, for the nth kinetic phase) using an iterative nonlinear least squares Levenberg-Marquardt algorithm (22).

### 4.4 Results

**The Reductive Half-reaction Kinetics of C30A Trimethylamine Dehydrogenase with Diethylmethylamine** - In order to investigate the effect of removal of the covalent 6-S-cysteinyi FMN bond on the reductive half-reaction of trimethylamine dehydrogenase,
the kinetics of the reaction of C30A mutant trimethylamine dehydrogenase with
diethylmethylamine has been examined. This slow substrate for native trimethylamine
dehydrogenase has the advantage that several well-resolved kinetic phases are observed
in the course of its reaction with enzyme (9). As with native trimethylamine
dehydrogenase, the reaction of the C30A mutant enzyme with diethylmethylamine at pH
8.0 exhibits three well-resolved kinetic phases (Fig. 4.1). The transient observed at 362
nm consists of only the two slower kinetic phases (Fig. 4.1A), while that observed at 410
nm consists of the two faster kinetic phases (Fig. 4.1B); the transient observed at 450 nm
contains all three kinetic phases (Fig. 4.1C). The observed rate constants for each kinetic
phase, designated as $k_{\text{fast}}$, $k_{\text{int}}$, and $k_{\text{slow}}$, respectively, are each independent of
observation wavelength. $k_{\text{fast}}$ exhibits hyperbolic dependence on the concentration of
diethylmethylamine and a fit of the data to the equation $k_{\text{obs}} = (k_{\text{lim}} [S])/(K_d + [S])$ (23)
gives values for $k_{\text{lim}}$ and $K_d$ of 80 s$^{-1}$ and 5.0 mM, respectively (Fig. 4.2). This $K_d$ (pH
8.0) is within experimental error of the value seen with native trimethylamine
dehydrogenase (4.3 mM) (9), whereas the value of $k_{\text{lim}}$ exhibited by C30A at pH 8.0 is
approximately six-fold smaller than that observed with native enzyme (Table 4.1).$^1$ $k_{\text{int}}$
and $k_{\text{slow}}$ for the C30A mutant are both independent of diethylmethylamine concentration
and have the values of 6 s$^{-1}$ and 0.2 s$^{-1}$, respectively, comparable to the values seen with
native enzyme (3.5 s$^{-1}$ and 0.2 s$^{-1}$). At pH 6, $k_{\text{lim}}/K_d$ for the fast kinetic phase is 0.05
mM$^{-1}$s$^{-1}$, 40-fold smaller than is observed for the native enzyme at the same pH (2.1 mM$^{-1}$
1s$^{-1}$), indicating that the removal of 6-S-cysteinyl FMN bond has a comparatively larger
effect as the pH is lowered.

Spectra for the Reaction Intermediates Seen in the Course of the Reaction of
C30A Trimethylamine Dehydrogenase with Diethylmethylamine - Spectra for the
intermediates seen in the course of the reaction of C30A trimethylamine dehydrogenase
with diethylmethylamine (Fig. 4.3B) have been calculated from the spectral changes

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Fig. 4.1. Transients observed for the reactions of oxidized C30A mutant trimethylamine dehydrogenase with diethylmethylamine. Absorbance changes observed at 362 nm (panel A), 410 nm (panel B), and 450 nm (panel C) after mixing in a stopped-flow apparatus are plotted versus time. Reaction conditions after mixing were: [C30A mutant enzyme] = 17 μM, [diethylmethylamine] = 2.5 mM, 0.1 M sodium pyrophosphate, pH 8.0, 25 °C. The symbols represent the data points and the solid lines represent fits of the data to the sum of two or three exponentials. Panel A, 362 nm. Data are fitted to the sum of two exponentials: $k_{\text{int}} = 6.0 \, \text{s}^{-1}$, $k_{\text{slow}} = 0.2 \, \text{s}^{-1}$. Panel B, 410 nm. Data are fitted to the sum of two exponentials: $k_{\text{fast}} = 28.2 \, \text{s}^{-1}$, $k_{\text{int}} = 6.0 \, \text{s}^{-1}$. Panel C, 450 nm. Data are fitted to the sum of three exponentials: $k_{\text{fast}} = 28.2 \, \text{s}^{-1}$, $k_{\text{int}} = 6.0 \, \text{s}^{-1}$, and $k_{\text{slow}} = 0.2 \, \text{s}^{-1}$.
Fig. 4.1

A. 362 nm

B. 410 nm
Figure 4.1 (continued)
Fig. 4.2. **Diethylmethylamine concentration dependence of the observed rate constants for the fast kinetic phase.** The observed rate constants for the fast kinetic phase at pH 8.0 are plotted versus the concentration of diethylmethylamine after mixing. The symbols represent \( k_{\text{fast}} \) measured at 450 nm. The solid line represents the fit of the data to the hyperbolic function: 
\[
    k_{\text{obs}} = k_{\text{lim}}[\text{diethylmethylamine}]/(K_d + [\text{diethylmethylamine}]).
\]
The fitted values for \( k_{\text{lim}} \) and \( K_d \) are 80 \( \text{s}^{-1} \) and 5.0 mM, respectively.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{lim}}$</th>
<th>$K_d$</th>
<th>$k_{\text{int}}$</th>
<th>$k_{\text{slow}}$</th>
<th>Ref.</th>
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</thead>
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<tr>
<td>Native TMADH</td>
<td>450 s$^{-1}$</td>
<td>4.3 mM</td>
<td>3.5 s$^{-1}$</td>
<td>0.2 s$^{-1}$</td>
<td>9</td>
</tr>
<tr>
<td>C30A TMADH</td>
<td>80 s$^{-1}$</td>
<td>5.0 mM</td>
<td>6.0 s$^{-1}$</td>
<td>0.2 s$^{-1}$</td>
<td>present study</td>
</tr>
</tbody>
</table>

Table 4.1 Comparison of kinetic parameters for the reactions of native and C30A TMADH with diethylmethylamine. The reaction conditions are: 0.1 M sodium pyrophosphate, pH 8.0, 25 °C.
Fig. 4.3. **Spectra for intermediates of the reaction of C30A mutant trimethylamine dehydrogenase with diethylmethylamine.** Reaction conditions after mixing in a stopped-flow apparatus were: [C30A mutant enzyme] = 9.6 μM, [diethylmethylamine] = 2.5 mM, 0.1 M sodium pyrophosphate, pH 8.0, 25 °C. Panel A, the kinetic difference spectra obtained from the absorbance changes associated with the fast kinetic phase (closed circles), the absorbance changes associate with the intermediate kinetic phase (open circles), and the absorbance changes associate with the slowest kinetic phase (closed triangles). Panel B: the absorbance spectra of oxidized C30A mutant enzyme (solid line), the intermediate formed by the fast kinetic phase (closed circles), the intermediate formed by the intermediate kinetic phase (open circles), and the final form at the end of the reaction (closed triangles). Panel C: deconvolution of the difference spectrum for the intermediate kinetic phase. The difference spectrum for flavin (flavin semiquinone-flavin hydroquinone) (open circles) is calculated by subtracting the difference spectrum for iron-sulfur center (reduced phenylhydrazine-treated trimethylamine dehydrogenase-oxidized phenylhydrazine-treated enzyme, taken from ref. 12) (closed triangles) from the difference spectrum for the intermediate kinetic phase (closed circles).
Fig. 4.3

![Absorbance vs Wavelength Graph]

Delta A

Absorbance

Wavelength (nm)

300 350 400 450 500 550
Figure 4.3 (continued)
associated with each of the three kinetic phases (Fig. 4.3A). The overall kinetic
difference spectrum obtained from the stopped-flow experiment is consistent with the
static difference spectrum observed upon the reduction of C30A mutant enzyme with
diethylmethylamine, indicating that there is no absorbance change in the dead time of the
stopped-flow apparatus. The absorbance change associated with the fast kinetic phase is
too small to be detected below 380 nm and decreases between 380 and 530 nm (Fig.
4.3A, closed circles). The spectral change for the fast phase seen with the C30A mutant
is quite different from that associated with the fast phase of the native enzyme (9) and is
typical of that observed upon reduction of normal FMN (as opposed to the 6-S-cysteiny1
FMN found in native trimethylamine dehydrogenase). These results indicate that the fast
phase represents bleaching of the enzyme FMN, presumably due to transient formation of
a substrate-flavin covalent adduct as has been proposed with native enzyme (9). The
limiting rate constant for this process is six-fold slower than is observed with the native
enzyme, indicating that formation of the covalent 6-S-cysteiny1 FMN bond facilitates this
first step of the reaction, but this step is not rate-limiting.

The difference spectrum for the intermediate phase (Fig. 4.3A, open circles),
which corresponds to intramolecular electron transfer from the flavin to the 4Fe-4S
center, is also different from that seen with the native enzyme in the region of 390-550
nm, again due to changes in the absorbance spectrum of the flavin upon loss of the
covalent linkage. The deconvolution of the spectral change for the intermediate phase
into components due to flavin hydroquinone→semiquinone and Fe/S_{ox}→Fe/S_{red} is
possible given that the Δε for the latter process is well defined (10), and the result is
shown in Fig. 3C. The difference spectrum for the 4Fe-4S center (closed triangles, taken
from ref. 10) is that observed upon reduction of phenylhydrazine-treated trimethylamine
dehydrogenase, a modified enzyme which contains a redox-inert 4a-phenylIFMN (4, 24).
The extinction change of the difference spectrum for the intermediate phase (closed
circles) was obtained using the concentration of the C30A mutant enzyme (9.6 μM) and 2 cm light path, taking into account the fact that only 30% of the C30A mutant enzyme contains flavin. The difference spectrum for one-electron oxidation of the reduced noncovalently bound FMNH2 (flavin semiquinone - flavin hydroquinone) (open circles) is obtained by subtracting the difference spectrum for 4Fe-4S center from that for the intermediate phase. The difference spectrum thus obtained has absorbance maxima at 360 and 410 nm, analogous to the ΔA seen for the semiquinone anion - hydroquinone of glucose oxidase (25), confirming that the flavin semiquinone formed with the C30A mutant enzyme is the flavin semiquinone anion, as is true of the native protein (26).

The spectral change associated with the slow phase (Fig. 4.3A, closed triangles) has the same shape as that for the intermediate phase (Fig. 4.3A, open circles). The same has been shown to be true with native trimethylamine dehydrogenase, and has been interpreted as indicating a shift in internal oxidation-reduction equilibrium toward full reduction of the 4Fe-4S center concomitant with formation of the spin-interacting state in this phase of the reaction.

**Formation of 6-OH FMN During Catalysis** - Upon reaction of the C30A mutant trimethylamine dehydrogenase with 10-fold excess of trimethylamine or diethylmethylamine (as in the above experiments) and passage through a G-25 column to reoxidize the enzyme and remove excess reagents, the absorbance spectrum shown in Fig. 4.4 (dotted line) is obtained, which is seen to differ significantly from that for the as-isolated C30A mutant enzyme (Fig. 4.4, solid line). The principal change is an absorption increase in the range of 300-450 nm and above 500 nm upon reaction with substrate. The substrate-treated C30A mutant enzyme cannot be reduced with substrate, and exhibits negligible activity.

Treatment of C30A mutant enzyme with 0.5 M perchloric acid followed by neutralization and centrifugation results in the release of normal FMN (data not shown).
Fig. 4.4. Spectra for as-isolated and substrate-treated C30A mutant trimethylamine dehydrogenase. The solid line is the spectrum for as-isolated C30A mutant. The dotted line is the spectrum for substrate-treated C30A mutant. All proteins are dissolved in 0.1 M potassium phosphate, pH 7.0.
The FMN content in C30A mutant enzyme thus determined is about 30% of the full complement, consistent with the known flavin content determined by other means (18). When the same procedure is applied to substrate-treated C30A mutant enzyme, however, a modified flavin is obtained. Fig. 4.5 shows the spectra for this modified FMN at pH 6 (solid line) and pH 10 (dotted line). The spectrum at pH 6 has an absorption maximum at 420 nm and a shoulder at 454 nm, and is identical to that for the neutral form of 6-hydroxyflavin (27-29). Similarly, the spectrum at pH 10 has absorption maxima at 424 and 584 nm and is the same as the spectrum for the anionic form of 6-hydroxyflavin (27-29). The pH dependence of the absorption spectrum (Fig. 4.5, inset) corresponds to a pKa of about 7, within experimental error of that (7.1) for authentic 6-hydroxyflavin (27-29). Taken together, these results make it clear that the modified flavin released from substrate-treated C30A mutant enzyme is 6-hydroxyFMN. To make sure that 6-hydroxyFMN is not the hydrolysis product of other flavin derivatives under the strongly acidic conditions of protein denaturation, substrate-treated C30A mutant protein at pH 7.0 was heated in boiling water for 3 min. Heat treatment also resulted in the release of 6-hydroxyFMN, indicating that 6-hydroxyFMN is formed during the treatment of C30A mutant with substrate and is not a product of hydrolysis in 0.5 M perchloric acid.

The most likely mechanism for formation of 6-OH FMN is via nucleophilic attack of OH− on the flavin C(6)-position. In order to determine more specifically how 6-hydroxyFMN is formed in C30A trimethylamine dehydrogenase, the mutant enzyme was reduced with dithionite at pH 7.0 and 10.0, passed through a G-25 column, and reoxidized in air. In neither case was 6-hydroxyFMN formed, indicating that OH− does not attack the flavin hydroquinone of reduced enzyme. Similarly, reduction of the C30A mutant enzyme with dithionite in the presence of 3 mM tetramethylammonium chloride, which results in two-electron reduction of the enzyme and formation of the spin-interacting state, does not give 6-hydroxyFMN, ruling out the possibility that OH− attacks
Fig. 4.5. **Spectra for neutral and anionic forms of 6-hydroxyFMN.** 6-hydroxyFMN is released from substrate-treated C30A mutant trimethylamine dehydrogenase by precipitation with perchloric acid. The solid line is the spectrum for neutral 6-hydroxyFMN (at pH 6.0). The dotted line is the spectrum for anionic 6-hydroxyFMN (at pH 10.0). The inset shows the plot of absorbance at 600 nm versus pH. The fit of the data gives a pKa of ~7.
flavin semiquinone or the spin-interacting state of the two-electron reduced C30A mutant. The data suggest that a reaction intermediate form generated in the course of the reductive half-reaction is particularly susceptible to nucleophilic attack.

It is of interest to know how many times the C30A mutant enzyme turns over before all the noncovalently bound FMN is converted to 6-hydroxyFMN. When C30A trimethylamine dehydrogenase is titrated with stoichiometric diethylmethylamine or trimethylamine at pH 7.0 and passed through a G-25 column, a spectrum similar to that for as-isolated C30A mutant was obtained, demonstrating that the noncovalently bound FMN is not quantitatively modified during the first turnover. In order to determine how many times the C30A mutant of trimethylamine dehydrogenase does turn over, enzyme assays utilizing ferricenium hexafluorophosphate as oxidant were carried out, following the reaction at 300 nm. The amount of ferricenium hexafluorophosphate reduced subsequent to the addition of an excess of diethylmethylamine was calculated using its extinction coefficient of 4300 M$^{-1}$cm$^{-1}$ at 300 nm. Because ferricenium hexafluorophosphate can accept only one reducing equivalent and substrate-reduced trimethylamine dehydrogenase possesses two reducing equivalents, the number of enzyme turnovers is obtained by dividing the amount of reduced ferricenium by two and by the amount of enzyme used. 1.8x10$^{-8}$ mole of C30A mutant enzyme can reduce only 3.37x10$^{-9}$ mole of ferricenium in the presence of excess of diethylmethylamine and ferricenium hexafluorophosphate, indicating that C30A mutant enzyme turns over approximately nine times before becoming inactive.

When C30A trimethylamine dehydrogenase is treated with excess of diethylmethylamine at pH 7.0, immediately passed through a G-25 column, and reoxidized in air, only part of the FMN is modified to 6-hydroxyFMN. When C30A mutant is incubated with excess of diethylmethylamine overnight, however, all the enzyme-bound FMN is modified. On the other hand, when trimethylamine is used
instead of diethylmethylamine and the mixture is immediately passed through a G-25 column, all the FMN is modified. These results indicate that the formation of 6-hydroxyFMN is slow when C30A mutant enzyme is treated with diethylmethylamine but faster if trimethylamine is used.

It is also of interest to see whether other nucleophiles could attack C(6) of the FMN in C30A mutant enzyme to form other flavin derivatives. When oxidized C30A mutant trimethylamine dehydrogenase is treated with sodium sulfide, a spectrum resembling that for 6-mercaptoflavin (30, 31) was obtained. The spectrum has absorption maxima at 428 and 632 nm (data not shown). This result suggest that the C(6) position is also susceptible to the nucleophilic attack by S2-. However, treatment of oxidized C30A trimethylamine dehydrogenase with potassium cyanide results in partial bleaching of the spectrum between 370 and 520 nm, but a spectrum similar to that for oxidized C30A mutant is obtained after reoxidation with air; treatment of C30A mutant protein with excess trimethylamine in the presence of potassium cyanide results in the formation of 6-hydroxyFMN. These results indicate that CN- cannot attack C(6) of FMN to form 6-CN-FMN. Similarly, treatment of oxidized C30A mutant with sodium sulfite and hydroxylamine hydrochloride in the absence or presence of trimethylamine did not result in the formation of flavin derivatives.

As is seen for native trimethylamine dehydrogenase, when C30A mutant enzyme is reduced with dithionite in the presence of tetramethylammonium chloride, the unpaired electron spins of the flavin semiquinone and reduced iron-sulfur center interact strongly to give rise to a spin-interacting state which exhibits an intense g=4 EPR signal (18). In order to determine whether the unpaired electron spin of 6-hydroxyFMN semiquinone can interact with that of the reduced iron-sulfur center, substrate-treated C30A mutant enzyme was reduced with dithionite in the presence of tetramethylammonium chloride. No g=4 EPR signal was observed, indicating that when FMN is modified to form 6-
hydroxyFMN, the unpaired electron spins of the two prosthetic groups cannot interact to
give a spin-interacting state.

4.5 Discussion

Similar to the reaction of native trimethylamine dehydrogenase with
diethylmethylamine, the reaction of the C30A enzyme with this slow substrate is found to
exhibit three kinetic phases, with only $k_{\text{fast}}$ dependent on the concentration of
diethylmethylamine. The spectral change associated with the fast phase represents loss of
the characteristic absorbance of the noncovalently bound, oxidized FMN upon reaction
with diethylmethylamine. The spectral change associated with the intermediate phase
indicates that this process represents the intramolecular electron transfer from flavin
hydroquinone to the iron-sulfur center, leaving flavin semiquinone anion. In the slow
phase a small amount of additional electron transfer occurs that is associated with the
spin-interacting state. The results suggest that the mechanism of the reaction of
diethylmethylamine with the C30A enzyme is fundamentally the same as its reaction with
native trimethylamine dehydrogenase. The $K_d$ for diethylmethylamine in the fast phase
of the reaction has the same value for both native and C30A mutant enzyme, indicating
that the 6-S-cysteinyl FMN bond has no effect on the substrate binding. On the other
hand, the value of $k_{\text{lim}}$ for the fast phase of the reaction of the C30A enzyme with
diethylmethylamine is approximately six-fold smaller than is seen with the native
enzyme, demonstrating that removal of the 6-S-cysteiny1 FMN bond slows down the first
step of the reductive half-reaction. The values for $k_{\text{int}}$ and $k_{\text{slow}}$ of the reaction of C30A
trimethylamine dehydrogenase with diethylmethylamine are comparable to those seen
with native enzyme, indicating that the removal of the 6-S-cysteiny1 FMN bond has
minimal affect on the later steps in the catalytic sequence. It is to be emphasized that the
first, mutation-sensitive step of the reductive half-reaction is not rate-limiting and so the kinetic effect of loss of covalent flavin attachment is minimal.

The present work demonstrates that the noncovalently bound FMN is modified to form 6-hydroxyFMN and the enzyme becomes inactive after the enzyme turns over on average nine times when C30A trimethylamine dehydrogenase turns over under steady-state conditions. It is thus evident that one significant role for the 6-S-cysteinyl FMN bond in native trimethylamine dehydrogenase is to prevent enzyme inactivation in the course of catalysis. The formation of 6-hydroxyFMN is not due to simple reduction of flavin in the course of catalysis, however, as the C30A enzyme is not modified upon reduction with excess dithionite at either pH 7.0 or 10.0. Similarly, formation of the spin-interacting state does not result in formation of 6-hydroxyFMN either since the C30A enzyme is unchanged upon reduction with dithionite in the presence of tetramethylammonium chloride.

The most likely mechanism whereby 6-hydroxyFMN is formed is via nucleophilic attack of HO\(^-\) at the flavin C(6) position in a manner similar to the cysteine thiolate attack that is thought to give rise to the covalent adduct of native enzyme (18). It is clear from the above, however, that it must be an intermediate formed in the course of the catalytic sequence that is susceptible to nucleophilic attack, and not simply oxidized or reduced flavin. We suggest that it is the covalent flavin-substrate adduct previously proposed (9) that is the form rendered susceptible to nucleophilic attack, as shown in Scheme 1.

The covalent flavin-substrate adduct being the form of flavin susceptible to nucleophilic attack also provides a rationale as to why formation of 6-hydroxyFMN occurs so much faster with trimethylamine as substrate rather than diethylmethylamine. It is possible that formation of the 6-hydroxyFMN in the course of steady-state assays accounts for the large apparent value of \(K_m\) observed with C30A trimethylamine dehydrogenase (18).
The x-ray crystal structure of trimethylamine dehydrogenase shows that the flavin ring is distinctly nonplanar, bending 20° about a line connecting N-5 and N-10 atoms of the isoalloxazine ring (13, 14). It has been proposed that the nonplanarity of the flavin ring may facilitate the reductive half-reaction of trimethylamine dehydrogenase by raising the flavin reduction potential and may be due in part to the 6-S-cysteinyl linkage(18). The results obtained in the present study demonstrate that the removal of the 6-S-cysteinyl FMN bond slows down the initial reaction of substrate with the enzyme-bound FMN, but not the overall reductive half-reaction because the rate of the rate-limiting step is unchanged. The modest effect seen for the C30A mutant enzyme on the limiting rate constant for the fast phase of the reaction with diethylmethylamine indicates that while the 6-S-cysteinyl FMN bond may increase the FMN reduction potential, the effect is not
profound from a kinetic standpoint. It appears instead that one major role of the covalent linkage is simply to prevent the formation of 6-hydroxyFMN in the active site of trimethylamine dehydrogenase so that it does not become inactive under multiple turnover conditions. In this sense, it is possible that the covalent attachment found in native trimethylamine dehydrogenase is more appropriately thought of as an effect of other factors related to the flavin binding site that renders (perhaps coincidentally) the C(6) position susceptible to nucleophilic attack, rather than a principal cause of the chemistry exhibited by the flavin that facilitates catalysis.

As is seen for native and recombinant wild-type trimethylamine dehydrogenase, the unpaired electron spins of the noncovalently bound flavin semiquinone and reduced iron-sulfur center can interact to give rise to a spin-interacting state (18), demonstrating that 6-S-cysteinyl FMN bond does not play an important role in the formation of spin-interacting state. When the ferredoxin-type 4Fe-4S center in native enzyme is modified to an EPR active iron-sulfur center by treatment of native enzyme with ferricenium hexafluorophosphate at pH 10, the unpaired electron spin of the modified iron-sulfur center cannot interact with that of the flavin semiquinone (see Chapter 5). Similarly, when the FMN in C30A mutant enzyme is modified to 6-hydroxyFMN, the unpaired electron spins of 6-hydroxyflavin semiquinone and reduced iron-sulfur center no longer interact. All these results suggest that both ferredoxin-type 4Fe-4S center and unmodified FMN are necessary for the formation of the spin-interacting state.
4.6 Footnote

1. The smaller limiting rate constant for C30A mutant enzyme is not due to the 30% of the full complement of FMN because the deflav enzyme is catalytically inert in the present experiments.
LIST OF REFERENCES


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CHAPTER 5

SPECTROSCOPIC CHARACTERIZATION OF FERRICENIUM-TREATED TRIMETHYLAMINE DEHYDROGENASE

5.1 Abstract

Ferricenium-treated trimethylamine dehydrogenase (TMADH) has been characterized with UV/Vis absorption, CD, EPR, Mössbauer, and MCD spectroscopies. After treatment with ferricenium hexafluorophosphate, a strong, chemically inert oxidant, at pH 10, a modified iron-sulfur center gives rise to an axial EPR signal with g-values of 2.04 and 2.01 which resembles the EPR spectra of *Rhodopseudomonas gelatinosa* high potential iron protein (HiPIP) and of 3Fe cluster in beef liver cytoplasmic aconitase. The new EPR signal cannot be saturated by up to 140 mW microwave power. The modified iron-sulfur center cannot be converted to a ferredoxin type 4Fe-4S center. The Mössbauer and MCD results show that the modified iron-sulfur center is neither a 3Fe cluster, nor a HiPIP 4Fe-4S center. Excess substrate trimethylamine can reduce the FMN, but not the modified iron-sulfur center in ferricenium-treated TMADH. When ferricenium-treated TMADH is reduced to one electron per subunit at pH 10, the modified iron-sulfur center does not interact with flavin semiquinone, suggesting that a
normal 4Fe-4S center is necessary for the formation of the spin-interacting state of native TMADH.
5.2 Introduction

Trimethylamine dehydrogenase (TMADH\textsuperscript{1}; EC 1.5.99.7) isolated from *Methylophilus methylotrophus* (former designated as W\textsubscript{3A}\textsubscript{1}) is a homodimer of molecular weight of 166,000 Da. Each subunit possesses a covalently bound 6-cysteinyl FMN coenzyme and a 4Fe-4S (ferredoxin-type) iron-sulfur center (1-4), and also one equivalent of tightly bound ADP (5). The enzyme catalyzes the oxidative demethylation of trimethylamine to dimethylamine and formaldehyde, passing the pair of reducing equivalents thus obtained to two equivalents of an electron-transferring flavoprotein (ETF), which becomes reduced to the level of the (anionic) semiquinone (4, 6-9). When TMADH is reduced to the level of two electrons per subunit by excess substrate, the magnetic moments of the unpaired electrons of the flavin semiquinone and reduced iron-sulfur center interact to give rise to a spin-interacting state which is distinguished by a complex EPR signal centered near $g$=2 and an unusually intense half-field ($g$~4) signal (10-17).

There are three oxidation states for 4Fe-4S cluster: [4Fe-4S]$^{3+}$, [4Fe-4S]$^{2+}$, and [4Fe-4S]$^{1+}$. The bacterial ferredoxin type 4Fe-4S cycles between the 2+ and 1+ states. In contrast, high potential iron protein (HiPIP) 4Fe-4S cycles between the 3+ and 2+ levels. The 4Fe-4S center in oxidized TMADH is a bacterial ferredoxin-type and in the diamagnetic (2+) state, and thus EPR-silent. When such a cluster is reduced from [4Fe-4S]$^{2+}$ to the 1+ state, it gives rise to a rhombic EPR signal with $g_{\text{ave}}$<2 (11). Oxidized HiPIP exhibits an axial EPR signal with $g_{\text{ave}}$>2 (23, 24), while reduced HiPIP is diamagnetic. When protein containing ferredoxin type [4Fe-4S]$^{2+}$ cluster is treated with oxidant, instead of obtaining [4Fe-4S]$^{3+}$ cluster, the 4Fe cluster is degraded to a 3Fe cluster which shows a similar EPR signal with $g_{\text{ave}}$>2 as HiPIP (24-26, 28, 29).

In this work, we report characterization of ferricenium-treated TMADH using UV/Vis absorption, CD, EPR, Mössbauer, and MCD spectroscopies.
5.3 Materials and Methods

*Enzyme Purification and Materials - Methylophilus methylo trophus* W3A1 was grown and trimethylamine dehydrogenase was purified as described by Steenkamp and Mallinson (18), 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 mM⁻¹cm⁻¹ (19). Phosphate buffer, trimethylamine hydrochloride, benzyl viologen, and phenazine ethosulfate were obtained from Sigma, and boric acid from Jenneil Chemical Co.. Sodium dithionite was obtained from Virginia Chemicals and phenyl hydrazine from Eastman Kodak. Ferricenium hexafluorophosphate was prepared as described by Lehman et al. (19, 20).

*Treatment of TMADH with Ferricenium Hexafluorophosphate* - TMADH in 0.1 M borate buffer, pH 10.0 was incubated with 3 mM ferricenium hexafluorophosphate at room temperature for 4 hrs. The solution was then centrifuged at 14,000 rpm for 15 mins to eliminate precipitated ferrocene and passed through a Sephadex G-25 column equilibrated with appropriate buffer. The concentration of ferricenium-treated TMADH was determined by the method described by Bradford (21). Iron analysis was done by the method described by Fish (22).

*Spectroscopic Measurements*-- Optical spectra were recorded at room temperature with a Hewlett-Packard 8452A single beam diode array spectrophotometer. Circular dichroism spectra were obtained over the range 350-800 nm at ambient temperature using an Aviv model 40DS vis-IR circular dichroism spectrophotopolarimeter. EPR samples were frozen by slow immersion in liquid nitrogen. X-band EPR spectra were recorded at 15 K using a Brüker ER 300 EPR spectrometer equipped with a ER035M gauss meter and a Hewlett-Packard 5352B microwave frequency counter. Instrument parameters are
given in the appropriate figure legends. A total of 5-10 40-second scans were accumulated for each sample to improve the signal-to-noise ratio. EPR signal intensities were determined by double integration of spectra using Bruker Instruments software. Quantitation of superoxidized iron-sulfur center was accomplished using the EPR signal of fully reduced native TMADH as an integration standard. Mössbauer experiments are performed at 175 °K.

5.4 Results

Absorption and CD Spectra of Ferricenium-treated TMADH - The absorption spectra of native and ferricenium-treated TMADH are presented in Fig. 5.1. When TMADH is treated with 3 mM ferricenium hexafluorophosphate at pH 10.0 and room temperature for 4 hrs, the absorbance between 300 - 400 nm increases while that above 400 nm decreases, especially in the range of 600-800 nm. Fig. 5.1 also shows the spectra for both trimethylamine and dithionite reduced forms of ferricenium-treated TMADH. The CD spectra of native and ferricenium-treated TMADH are shown in Fig. 5.2. Ferricenium-treated TMADH gives a different CD signal than does native TMADH in the range of 350 -460 nm. The CD spectral change upon treatment of TMADH with ferricenium hexafluorophosphate is consistent with the absorption spectral change shown in Fig. 5.1. The CD signal increases between 350 and 380 nm, while that between 380 and 470 nm decreases.

EPR studies of Ferricenium-treated TMADH - The 4Fe-4S center in the oxidized native TMADH is in the diamagnetic [4Fe-4S]$^{2+}$ state and is EPR-silent. After TMADH was treated with 3 mM ferricenium hexafluorophosphate at pH 10.0 and room temperature for 4 hrs, a new EPR signal attributable to the ferricenium-treated enzyme
Fig. 5.1. *Native and ferricenium-treated TMADH optical spectra.* The spectra shown are for native enzyme (-----), ferricenium-treated TMADH (solid line), trimethylamine reduced, ferricenium-treated TMADH (.......), and dithionite reduced, ferricenium-treated TMADH (---) in 0.1 M potassium phosphate buffer, pH 7.0.
Fig. 5.2. *Native and ferricenium-treated TMADH CD spectra.* The spectra shown are for native enzyme (dashed line) and ferricenium-treated TMADH (solid line) in 0.1 M potassium phosphate buffer, pH 7.0.
Fig. 5.3. EPR spectra of oxidized and dithionite reduced ferricenium-treated TMADH. Panel A: ferricenium-treated TMADH was dissolved in 0.1 M potassium phosphate buffer, pH 7.0. Panel B: ferricenium-treated TMADH in 0.1 M potassium phosphate buffer, pH 7.0, was reduced with excess dithionite in the presence of 5 μM benzyl viologen and phenazine ethosulfate, passed through a G-25 column to get rid of dyes, and reduced again with dithionite. Spectrometer parameters for panel A and B: microwave frequency, 9.4571 GHz; microwave power, 1.00 milliwatts; modulation amplitude, 10.084 gauss, 15 °K.
B (continued)
arised (Fig. 5.3A). To make sure this new signal was not caused by the binding of ferricenium or ferrocene to the enzyme, ferricenium-treated TMADH was passed through a phenyl-sepharose column (in addition to the G-25 column used to separate enzyme from the initial reaction mixture) and an EPR spectrum was recorded. The new EPR signal did not disappear after the phenyl-sepharose column step, indicating that the new signal was due to oxidation and/or chemical modification of the enzyme. The new EPR signal, however, disappeared at liquid nitrogen temperature (150 °K), suggesting that it arises from the modified iron-sulfur center, not a radical. The new EPR signal with g-values of 2.04 and 2.01 is similar to those of *Rhodopsseudomonas gelatinosa* HiPIP (23) and of 3Fe cluster in beef liver cytoplasmic aconitase (24). Double integration of this new signal showed that the iron-sulfur center was quantitatively converted to this paramagnetic species. Power saturation experiments showed that the EPR signal shown in Fig. 5.3A could not be saturated by up to 140 mW microwave power (Fig. 5.4). When TMADH was treated with 3 mM ferricenium hexafluorophosphate at pH 8.0 and 6.0 for 4 hrs, only ~60% and ~6% of the iron-sulfur center, respectively, was converted to the paramagnetic species. Ferricyanide was not effective in generating the new EPR signal.

When ferricenium-treated TMADH was reacted with excess trimethylamine at pH 7.0, the absorbance between 400 - 650 nm decreased as the enzyme flavin became reduced (Fig. 5.1), but the intensity of the new EPR signal shown in Fig. 5.3A did not diminish. These results indicate that trimethylamine cannot reduce the modified iron-sulfur center. Furthermore, when ferricenium-treated TMADH was reduced with excess dithionite, the intensity of the EPR signal shown in Fig. 5.3A decreased but did not disappear, indicating that excess dithionite does not effectively reduce the modified iron-sulfur center. In the presence of benzyl viologen and phenazine ethosulfate, however, dithionite can further reduce ferricenium-treated TMADH. After treatment with
Fig. 5.4. EPR power saturation profile. Double integration intensity of EPR signals of ferricenium-treated TMADH was plotted versus the square root of the applied microwave power at 15 °K. The symbols represent the intensity which is normalized to the intensity at 138 mW microwave power. Spectrometer parameters are the same as those in Fig. 5.3 except modulation amplitude is 5.054 gauss.
dithionite in the presence of benzyl viologen and phenazine ethosulfate, passage through a G-25 column to get rid of dyes, and treatment with dithionite again, ferricenium-treated TMADH still gave rise to an EPR signal (Fig. 5.3B) which could be seen even at liquid nitrogen temperature (150 °K) that was neither the new EPR signal nor that for the reduced 4Fe-4S center of untreated enzyme (11).

When native TMADH is reduced with dithionite to the level of two electrons per subunit at pH 10, the flavin semiquinone and reduced iron-sulfur center interact each other to give rise to a complex EPR signal centered near g=2 and an intense half-field (g~4) signal (17). It is of interest to see whether the modified iron-sulfur center can interact with flavin semiquinone or not. To do so, ferricenium-treated TMADH was reduced to one electron per subunit at both pH 7.0 and 10.0. The EPR signal for modified iron-sulfur center and flavin semiquinone were seen at 15 °K and 40 °K, respectively, but neither complex EPR signal at g=2 nor half-field signal was detected at both pH and 15 °K, indicating that the modified iron-sulfur center can not interact with flavin semiquinone.

It has been reported that oxidation of Clostridium pasteurianum ferredoxin or Bacillus subtilis glutamine phosphoribosylpyrophosphate amidotransferase with ferricyanide causes degradation of 4Fe-4S clusters to 3Fe clusters (24-26), and the 3Fe clusters thus formed can be converted to 4Fe-4S clusters by reduction with dithionite and addition of sulfide or Fe(II) (24-27). Ferricenium-treated TMADH was reduced with dithionite, treated with Fe(II) and/or sulfide, and reduced with dithionite to see if a 4Fe-4S cluster could be reconstituted. No rhombic EPR signal was obtained, indicating that superoxidized iron-sulfur center cannot be converted back to a normal [4Fe-4S]^{2+} center in the present condition. In addition, iron quantitation indicated that treatment of TMADH with ferricenium did not significantly decrease the iron content of the enzyme:

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ferricenium-treated and native TMADH contained 3.3±0.2 and 3.5±0.2 Fe per monomer, respectively.

Mössbauer and MCD studies of Ferricenium-treated TMADH - Because EPR spectrum cannot be used as a criterion to distinguish [4Fe-4S]$^{3+}$ from 3Fe clusters (24), Ferricenium-treated TMADH was further characterized using Mössbauer and MCD spectroscopy. The Mössbauer spectra of native and ferricenium-treated TMADH are shown in Fig. 5.5 and the isomer shifts ($\delta$/Fe) and quadrupole splittings (QS) are given in Table 5.1. The solid lines in Fig. 5.5 represent simulations with Lorentzian lineshapes.

For native TMADH, the intensity ratio of the peaks is one in the minor peak to three in the major peak. But the intensity ratio of the peaks for ferricenium-treated TMADH is one to six. The Mössbauer spectrum for ferricenium-treated TMADH is quite different from those for 3Fe center (24) and HiPIP 4Fe-4S center (31), indicating that the modified iron-sulfur center in ferricenium-treated TMADH is neither a 3Fe center nor a HiPIP 4Fe-4S center. Preliminary MCD results (data not shown) also suggest that the modified iron-sulfur center is not a 3Fe center or a HiPIP iron-sulfur center.

<table>
<thead>
<tr>
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<th>Native TMADH</th>
<th>Ferricenium-treated TMADH</th>
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<tbody>
<tr>
<td></td>
<td>$\delta$/Fe</td>
<td>QS</td>
</tr>
<tr>
<td>Minor peak</td>
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<td>0.72</td>
</tr>
<tr>
<td>Major peak</td>
<td>0.41±0.04</td>
<td>1.35±0.14</td>
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Table 5.1 Mössbauer parameters of native and ferricenium-treated TMADH at 175 °K
Fig. 5.5 Mössbauer spectra for native and ferricenium-treated TMADH at 175 °K. Top spectrum: native TMADH. Bottom spectrum: ferricenium-treated TMADH.
5.5 Discussion

Treatment of TMADH with ferricenium hexafluorophosphate at pH 10 results in the appearance of an axial EPR signal with g-values of 2.04 and 2.01 which arises from the modified iron-sulfur center. The new EPR spectrum resembles those for a HiPIP \([4\text{Fe}-4\text{S}]^{3+}\) and a 3Fe center (23, 24). When ferricenium-treated TMADH is reduced with dithionite, however, no rhombic EPR signal for \([4\text{Fe}-4\text{S}]^{1+}\) has been observed. Moreover, the modified iron-sulfur center cannot be converted to \([4\text{Fe}-4\text{S}]^{2+}\). These results indicate that the modified iron-sulfur center is neither a 3Fe center nor a HiPIP-type \([4\text{Fe}-4\text{S}]^{3+}\) center. The Mössbauer and MCD results further confirm this conclusion. The results at present, however, are inconclusive.

The catalytic mechanism of TMADH has been investigated (9,15,17). The FMN first takes up two electrons from substrate and then transfers one electron to the iron-sulfur center. The electrons was then passed to ETF via the iron-sulfur center. After TMADH is treated with ferricenium hexafluorophosphate, the FMN can still be reduced by substrate, but the electrons cannot be transferred to the modified iron-sulfur cluster and thus cannot be passed to ETF. Thus, treatment with ferricenium hexafluorophosphate inactivates TMADH by rendering the enzyme non-functional in the oxidative half-reaction.

The optical absorption spectrum for ferricenium-treated TMADH has less absorption between 600 nm and 800 nm than that for the untreated enzyme, demonstrating that the bulk of the absorption between 600 nm and 800 nm in the native TMADH spectrum has contribution from the normal 4Fe-4S center. Trimethylamine can reduce the FMN, but not the modified iron-sulfur center in ferricenium-treated TMADH, indicating that treatment with ferricenium hexafluorophosphate has little effect on the FMN. When ferricenium-treated TMADH is reduced with dithionite to one electron per
subunit at pH 10, the modified iron-sulfur center cannot interact with the flavin semiquinone, suggesting that 4Fe-4S center is necessary for the formation of the spin-interacting state of native enzyme.
5.6 Footnotes

1. The abbreviations used are: TMADH, trimethylamine dehydrogenase; 4Fe-4S, four iron-four sulfur center; HiPIP, high potential iron protein.
LIST OF REFERENCES


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