Intramolecular Electron Transfer in Trimethylamine Dehydrogenase: A Thermodynamic Analysis†

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ABSTRACT: Within the enzyme trimethylamine dehydrogenase [TMADH], intramolecular electron transfer occurs between a fully reduced covalently bound 6-*S*-cysteinylflavin [FMN] cofactor, and an oxidized iron−sulfur $[4Fe\text{-}4S]^{2+}$ center. When the enzyme is reduced by the substrate trimethylamine, the kinetics of this intramolecular electron transfer [ET] reaction are biphasic, suggesting that ET ocurs via two alternative processes [Falzon, L., & Davidson, V. L. (1996) *Biochemistry 35*, 2445−2452]. The formation of the FMN semiquinone was monitored by stopped-flow spectroscopy, and the two rate constants for the biphasic reaction were determined at temperatures ranging from 12 to 37 °C. Analysis of these rate constants by ET theory yielded values of 2.2 eV for the reorganizational energy [λ] associated with each reaction and electronic couplings [H_{AB}] of 5.9 and 47 cm^{-1} for the slower and faster ET reactions, respectively. The analysis also predicted average theoretical distances between the two redox centers of 12.3 Å for the slower reaction and 8.1 Å for the faster reaction. These predicted distances correlate well with the known crystal structure of TMADH and the most efficient pathways for ET that were predicted from the known structure using the Greenpath program. This analysis suggests that for each reaction the ET event is rate-limiting, but coupled to a highly unfavorable non-ET process, and that binding of a second molecule of substrate to reduced TMADH decreases the efficiency of the intramolecular ET.

Trimethylamine dehydrogenase (TMADH),[1] an enzyme from the Gram-negative restricted facultative methylotrophic bacterium *Methylophilus methylotrophus* sp. W3A1, catalyzes the oxidative N-demethylation of trimethylamine to dimethylamine and formaldehyde (Colby & Zatman, 1974). The enzyme is a homodimer of M_r 166 kDa. Each subunit contains two cofactors, an unusual covalently bound 6-*S*-cysteinylflavin mononucleotide (FMN) (Steenkamp et al., 1978b), and a single iron−sulfur cluster, [4Fe-4S] (Hill et al., 1977), which are involved in catalysis and the subsequent ET to its physiological electron acceptor, electron transfer flavoprotein (Steenkamp & Gallup, 1978; Davidson et al., 1986). Pronounced differences have been observed between dithionite- and substrate-reduced TMADH by both EPR and optical spectroscopy. When TMADH is reduced by dithionite, three electrons are taken up by the enzyme, while when reduced by substrate only two electrons are taken up. Initially the two electrons reside on the FMN cofactor, and then one of the electrons is transferred intramolecularly to the iron−sulfur center. The initial reduction of FMN by substrate occurs rapidly, and the subsequent reduction of the [4Fe-4S] center occurs more slowly and exhibits complex kinetics (Steenkamp et al., 1978a,b; Bellamy et al., 1989; Rohlfs & Hille, 1994; Falzon & Davidson, 1996). The relative slowness of the rate of intramolecular ET from $FMNH_2$ to $[4Fe\text{-}4S]^{2+}$ is interesting given the close proximity of the two redox centers. The crystal structure of TMADH indicates that the 8α-methyl carbon of FMN is about 6 Å from the nearest iron atom. Furthermore, it was shown in pH-jump experiments (Rohlfs & Hille, 1991) that the reduction of $[4Fe\text{-}4S]^{2+}$ by dithionite-reduced $FMNH_2$ in the absense of substrate is significantly faster, suggesting that bound substrate or product somehow reduces the observed rate of ET from $FMNH_2$.

In a previous study we examined the steady-state and transient kinetic properties of TMADH and proposed a model [Scheme 2 in Falzon and Davidson (1996)] in which TMADH binds two molecules of trimethylamine, one which is converted to products and one which is not. Binding of the latter exerts profound effects on the activity of the enzyme. The kinetics of the intramolecular ET reaction from $FMNH_2$ to $[4Fe\text{-}4S]^{2+}$ are biphasic. The faster rate dominates at low substrate concentration and the slower rate dominates at high substrate concentration. In other words, the amplitude of the absorbance change associated with the faster reaction decreases with increasing substrate concentration and the amplitude of the absorbance change associated with the slower reaction increases with increasing substrate concentration (Falzon & Davidson, 1996). We characterized the manner by which excess substrate inhibits the steady-state turnover of TMADH and proposed that it may act as a switch between alternative mechanisms that regulate the rate of intramolecular ET reaction. As discussed previously (Falzon & Davidson, 1996), this may be a physiologically relevant mechanism for avoiding the overproduction of the potentially toxic formaldehyde product under conditions where formaldehyde production by TMADH might exceed the capacity of the cell to assimilate this product. To further test our kinetic model and to obtain information on how these

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[1] Abbreviations: TMADH, trimethylamine dehydrogenase; TMAC, tetramethylammonium chloride; FMN, flavin mononucleotide; ET, electron transfer.

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processes are regulated, we have performed a kinetic and thermodynamic analysis of the two alternative intramolecular ET reactions from $FMNH_2$ to $[4Fe\text{-}4S]^{2+}$ in TMADH. The structure of TMADH has already been determined by X-ray crystallography (Lim et al., 1986; Barber et al., 1992), and its amino-acid sequence is known (Boyd et al., 1992). This has allowed us to correlate the results of the thermodynamic analysis with the known crystal structure of TMADH and a Pathways (Regan et al., 1993) analysis of potential ET paths from the FMN cofactor to the iron−sulfur center within the enzyme. In this paper we report values for the thermodynamic parameters which govern each ET reaction and use ET theory to predict from the solution studies the distances for the ET reactions. These predicted distances correlate quite well with the structural data and suggest that these reactions are not gated, even though the observed ET rates intutively appear to be too slow for a true ET reaction.

EXPERIMENTAL PROCEDURES

Protein Purification. *Methylophilus methylotrophus* sp. W3A1 (NCIB 11348) was grown on the minimal medium of Owens and Keddie (1969) using 0.5% dimethylamine as a carbon source. Extraction and purification of TMADH were as described previously (Falzon & Davidson, 1996). Enzyme concentrations were determined from the absorbance of the oxidized enzyme at 443 nm using an extinction coefficient of 54 600 M^{-1} cm^{-1} (Kasprazak et al., 1983) in 0.1 M potassium phosphate buffer, pH 7.5.

Kinetic Analysis. Stopped-flow experiments were performed essentially as previously described (Falzon & Davidson, 1996) using an On-Line Instrument Systems (OLIS, Bogart, GA) RSM 1000 stopped-flow spectrophotometer. Data were collected and analyzed using OLIS software on an IBM compatible 486 personal computer. We have observed that the reduced forms of TMADH are quite stable against reoxidation by air, and, therefore, anaerobic conditions were not necessary for these experiments. TMADH (2−4 μM) was mixed with various concentrations of trimethylamine (50 μM to 4 mM) in 0.1 or 0.01 M potassium phosphate buffer, pH 7.5, at different temperatures. The rate of intramolecular ET (monitored by the change in absorbance at 365 nm) was slow enough under these conditions to be measured by the stopped-flow technique. Under these conditions the varied substrate was in excess of the concentration of the fixed reactant, oxidized TMADH. Under these pseudo-first-order conditions, all absorbance changes could be fit to a two-exponential decay equation (eq 1), where ${}^{\text{fast}}k$

$$A_{365} = C_1(1 - e^{-{}^{\text{fast}}kt}) + C_2(1 - e^{-{}^{\text{slow}}kt}) + b \qquad (1)$$

and ${}^{\text{slow}}k$ are the observed rate constants for the faster and slower rates, respectively, associated with the formation of flavin semiquinone. C_1 and C_2 are related to the initial absorbance, and b is an offset value to account for a nonzero base line. The biphasic behavior at this wavelength was observed throughout the range of trimethylamine concentrations used. The k_{obs} for each reaction was determined from the average of at least three measurements. Nonlinear curve fitting of the data was performed with the Olis Software and the Sigma Plot 5.1 (Jandel Scientific, San Rafael, CA) computer program.

Analysis of Thermodynamic Data. The temperature dependence of the apparent ET rate constants was analyzed by two different methods. The data obtained for each independent rate constant were fit to the Eyring equation (eq 2), a specialized form of transition-state theory,

$$\ln\left(\frac{k_3 h}{k_B T}\right) = \frac{\Delta H^*}{RT} + \frac{\Delta S^*}{R} \qquad (2)$$

where h is the Planck's constant, R is the gas constant, T is the temperature, k_B is Boltzmann's constant, ΔS^* is activation entropy, and ΔH^* is activation enthalpy. The data obtained for each independent rate constant were also fit to eqs 3 and 4, which describe non-adiabatic ET reactions and which were derived in part from Marcus theory (Marcus & Sutin, 1985).

$$k_{ET} = [k_o e^{-\beta(r-r_o)}] e^{-(\Delta G^\circ + \lambda)^2/4\lambda RT} \qquad (3)$$

$$k_{ET} = \frac{4\pi^2 H_{AB}^2}{h\sqrt{4\pi\lambda RT}} e^{-(\Delta G^\circ + \lambda)^2/4\lambda RT} \qquad (4)$$

H_{AB} is the electronic coupling between redox centers and describes the degree of wavefunction overlap occurring between donor and acceptor sites. λ is the reorganizational energy. h is Planck's constant. R is the gas constant. T is the temperature. ΔG° is the standard free energy difference for the reaction. k_o is the characteristic frequency of the nuclei which is assigned a value of 10^{13} s^{-1}. r is the distance between donor and acceptor. r_o is the close contact distance (3 Å). β is the electronic decay factor which is related to the nature of the intervening medium between redox centers (Moser et al., 1992; Onuchic et al., 1992).

RESULTS

Stopped-Flow Kinetic Studies. Values of k_{obs} for both slow and fast phases of the intramolecular ET were obtained at different temperatures from 12 to 37 °C at varying trimethylamine concentrations with a fixed concentration of TMADH (Figure 1). For each temperature the change in absorbance at 365 nm, which corresponds to formation of the FMN semiquinone, was biphasic. It was assumed that each of the two observed rate constants, ${}^{\text{slow}}k$ and ${}^{\text{fast}}k$, obeyed the simple kinetic model given in eq 5 and that the biphasic behavior is because of two alternative mechanisms by which the formation of flavin semiquinone occurs.

$$S + FMN/[4Fe\text{-}4S]^{2+} \overset{K_d}{\rightleftharpoons} P^*FMNH_2/[4Fe\text{-}4S]^{2+} \overset{k_3}{\underset{k_4}{\rightleftharpoons}} P^*FMN_{semi}/[4Fe\text{-}4S]^{+} \qquad (5)$$

In this scheme, k_3 is the forward rate constant of the ET reaction and k_4 is the rate of the reverse reaction, if any. This model was chosen to incorporate the observations that each k_{obs} is dependent on substrate concentration and that in each case we are strictly monitoring the spectral change associated with the conversion described by k_3 in eq 5. For all temperatures, ${}^{\text{slow}}k$ and ${}^{\text{fast}}k$ showed a hyperbolic concentration dependence on concentration (Figure 1), indicating a two-step process in which the initial step(s) equilibrates much faster than the following ET step. The data for the concentration dependence of both the slow and fast observed rate constants were analyzed by eq 6 (Strickland et al., 1975).

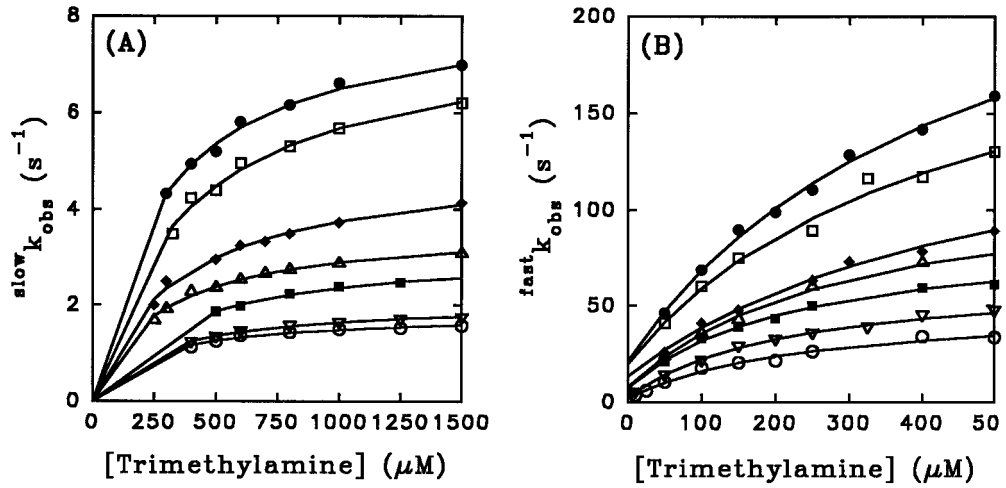


FIGURE 1: Concentration and temperature dependence of $^{slow}k_{obs}$ (A) and $^{fast}k_{obs}$ (B). The concentration of TMADH used in these experiments was 2−4 μM. All reactions were performed in 100 mM potassium phosphate buffer, pH 7.5. The temperatures at which each set of experiments was performed are as follows: (○) 285 K, (▽) 289 K, (■) 294 K, (△) 299 K, (◆) 303 K, (□) 307 K, and (●) 310 K. The solid lines represent the fits of each data set to eq 6.

$$k_{obs} = \frac{k_3[\text{trimethylamine}]}{[\text{trimethylamine}] + K_d} + k_4 \qquad (6)$$

Each kinetically determined K_d value is obviously not a true K_d (see eq 5). They reflect not only the initial binding of substrate but also other steps involved in the FMN reduction which precedes the one-electron transfer from $FMNH_2$ to $[4Fe\text{-}4S]^{2+}$. The values of k_3 and k_4 obtained for the faster phase and the values of k_3 obtained for the slower phase at each temperature are given in Table 1.

Finite values of the reverse process of the fast phase of the intramolecular ET, $^{fast}k_4$, were observed at all of the temperatures studied. Thus, for the faster phase it is possible to calculate an equilibrium constant for the reaction from the ratio of $^{fast}k_3/^{fast}k_4$ and to compare this value with that predicted from the redox potentials of the two redox centers. It has been previously reported (Barber et al., 1988) that the potential difference between the $FMNH_2/FMN_{semi}$ couple and the $[4Fe\text{-}4S]^{2+}/[4Fe\text{-}4S]^{+}$ couple in TMADH is +66 mV at 25 °C. This predicts that the equilibrium constant for the redox reaction at 25 °C will be equal to 13.1. The ratio of the experimentally determined $^{fast}k_4$ and $^{fast}k_3$ at 25 °C yields a measured equilibrium constant of 14.6, which corresponds to a potential difference of +69 mV and is in good agreement with the predicted value. This is consistent with the values of $^{fast}k_3$ describing rates of ET between the cofactors, which is dependent upon these redox potentials, rather than some non-ET event which is gating the ET reaction, and would not be related to the potential difference.

Unlike the fast phase, the slow phase ($^{slow}k_3$) appears to be irreversible. This is not necessarily unexpected. Steenkamp and Beinert (1982a) demonstrated that tetramethylammonium chloride (TMAC), which protects TMADH against inactivation by suicide inhibitors but does not reduce the enzyme, is able to bind to a form of TMADH other than the oxidized form. TMADH was first reduced with stoichiometric amounts of trimethylamine, and subsequent addition of TMAC resulted in spectral changes similar to those which characterize the enhancement of the triplet state upon addition of excess trimethylamine. According to Pace and Stankovich (1991), on binding of TMAC to TMADH the redox potentials of the $FMNH_2/FMN_{semi}$ and $[4Fe\text{-}4S]^{2+}/[4Fe\text{-}4S]^{+}$ couples are perturbed and the potential difference increases to +110 mV. Under these conditions, the formation of semiquinone will be essentially irreversible ($K_{eq} = 73$). It is possible that the redox potentials of the two cofactors in the presence of TMAC mimics that of the enzyme interacting with excess substrate (the enzyme form responsible for ^{slow}k). Thus, the fitted k_4 values of zero (Figure 1A) for the slow phase are consistent with ^{slow}k describing an intramolecular ET event when a second molecule of substrate is bound to TMADH.

Table 1: Kinetic Parameters for Intramolecular Electron Transfer Reactions in TMADH[a]

temperature (K)	$^{slow}k_3$ (s^{-1})	$^{fast}k_3$ (s^{-1})	$^{fast}k_4$ (s^{-1})
285	1.8 ± 1.4	48.3 ± 4.4	1.7 ± 1.4
289	2.1 ± 0.4	63.6 ± 2.5	2.4 ± 1.3
294	3.0 ± 0.7	80.9 ± 2.1	7.2 ± 2.7
299	3.7 ± 0.5	113 ± 6	7.7 ± 6
303	5.1 ± 0.5	154 ± 9	13.5 ± 3.1
307	7.7 ± 1.5	203 ± 40	19.7 ± 10.6
310	8.3 ± 1.3	260 ± 24	21.0 ± 5.6

[a] All parameters were obtained from fits of the data sets shown in Figure 1 to eq 6. Fitted values of $^{slow}k_4$ were essentially zero.

While they do appear to be dependent upon the redox potential difference between the cofactors, $^{slow}k_3$ and $^{fast}k_3$ should be considered apparent ET rate constants for the time being, because in the kinetic model any spectroscopically invisible reaction steps which precede the ET step may be reflected in these rates. These rates will only be true ET rates if no such additional steps occur or if the ET event is rate-limiting in a multistep mechanism where all other steps are energetically favorable (Hoffman & Ratner, 1987; Brunschwig & Sutin, 1989; Harris et al., 1994). To determine whether these rates describe a true ET event, or to what extent they may be influenced by some non-ET prerequisite step, we examined the temperature dependence and ionic strength dependence of each of these limiting first-order apparent ET rate constants.

Thermodynamic Analysis of the k_3. The temperature dependencies of $^{slow}k_3$ and $^{fast}k_3$ were analyzed by traditional transition-state theory, which provides information about the conversion of reactants into products in an adiabatic reaction. The data in Table 1 were fit to the Eyring equation (eq 2). The plots of ln $^{slow}k_3$ against $1/T$ and ln $^{fast}k_3$ against $1/T$ were

Table 2: Thermodynamic Parameters for Intramolecular Electron Transfer Reactions in TMADH

parameter[a]	rate constant	
	$^{slow}k_3$	$^{fast}k_3$
ΔS^* (J mol^{-1} K^{-1})	-85 ± 10	-50 ± 6
ΔH^* (kJ mol^{-1})	44 ± 3	46 ± 2
λ (eV)	2.2 ± 0.2	2.2 ± 0.1
H_{AB} (cm^{-1})	5.9 ± 4	47 ± 27
r (Å)	9.2−15.4[b]	6.4−9.7[b]

[a] The parameters were determined from the k_3 values obtained from the analysis of the data shown in Figure 1. ΔH^* and ΔS^* were determined from fits of the data to eq 2, λ and H_{AB} were determined from fits to eq 4, and r was determined from fits to eq 3. [b] The range of values for r is for fits of the data to eq 3 using a range of β values from 1.4 to 0.7 Å^{-1}.

each linear (plots not shown). The fitted values for the activation entropies and enthalpies for each rate constant are given in Table 2. The two reaction rates exhibited essentially identical activation enthalpies. The activation entropy for $^{slow}k_3$ was, however, significantly more negative than that for $^{fast}k_3$. This is consistent with $^{slow}k_3$ and $^{fast}k_3$ describing two different processes. If $^{slow}k_3$ and $^{fast}k_3$ are each really describing an ET event, then the interpretation of these parameters is not straightforward, as this reaction is nonadiabatic.

In a nonadiabatic reaction achievement of the transition-state energy does not always lead to product formation (Marcus & Sutin, 1985). Long-range ET reactions are nonadiabatic and have questionable reaction coordinates. Because they occur within the protein matrix they are collisionless. Nonadiabatic ET can be appropriately described by eqs 3 and 4, where the standard activation entropy and enthalpy changes are described in terms of λ and H_{AB}. The temperature dependence of $^{slow}k_3$ and $^{fast}k_3$ was analyzed by eqs 3 and 4 (Figure 2 and Table 2). This analysis of $^{slow}k_3$ and $^{fast}k_3$ yielded relatively large values of λ but values of H_{AB} which were within the nonadiabatic limit (discussed later). The value of H_{AB} associated with $^{fast}k_3$ was greater than that associated with $^{slow}k_3$, suggesting that the differences in the ET rates may be due to differences in the electronic coupling between the redox centers caused by binding of the second molecule of trimethylamine to TMADH. Analysis of $^{slow}k_3$ by eq 3 yielded a range of fitted values for the distance between the two cofactors of 9.2−15.4 Å, depending upon the β value that was used. This range was obtained using β values ranging from 1.4 to 0.7 Å^{-1} (Moser et al., 1992; Onuchic et al., 1992). The value of 0.7 is thought to be appropriate for electron tunneling through σ bonds in a covalently coupled system, while the value of 1.4 has previously been applied for ET through a protein matrix and is a value midway between those for covalently linked systems and a vacuum (Moser et al., 1992). For $^{fast}k_3$, the range of fitted values obtained for the distance was 6.4−9.7 Å. What this means is that if the average β values which

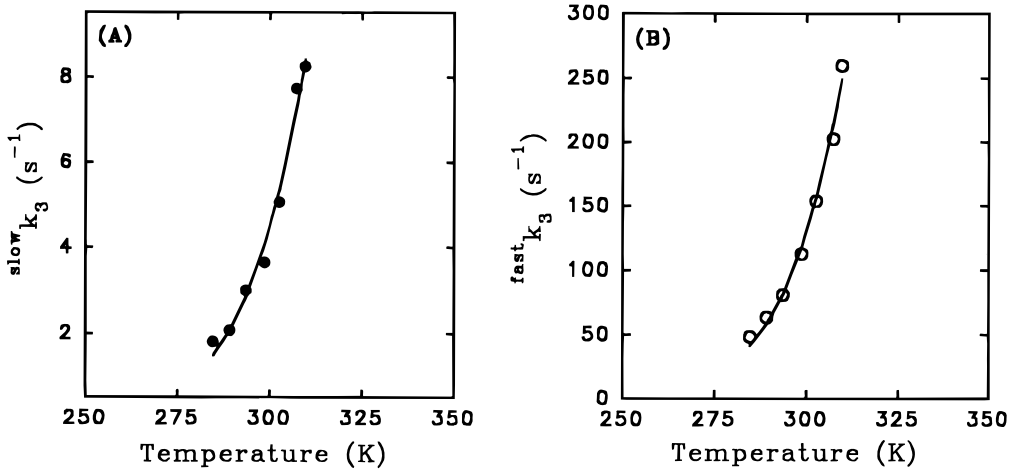


FIGURE 2: Analysis of the temperature dependence of $^{slow}k_3$ (A) and $^{fast}k_3$ (B). Values of k_3 were determined from the data shown in Figure 1. The solid lines represent the fits of each data sets to eqs 3 and 4. In each case the fits to eqs 3 and 4 are superimposable.

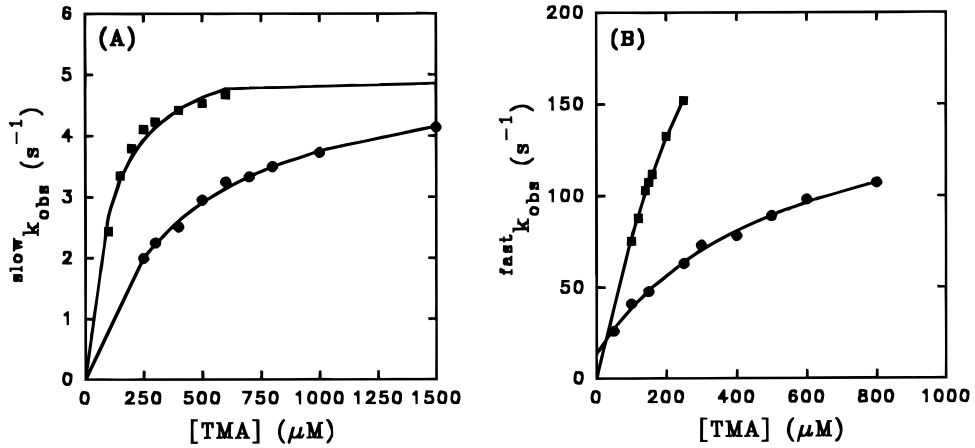


FIGURE 3: Ionic strength dependence of the k_3 rate constants. The concentration dependence of $^{slow}k_3$ (A) and $^{fast}k_3$ (B). Reactions were performed in 100 mM potassium phosphate buffer, pH 7.5 at 30 °C (●), and in 10 mM potassium phosphate buffer, pH 7.5 at 30 °C (■). The solid lines represent the fits of each data set to eq 6.

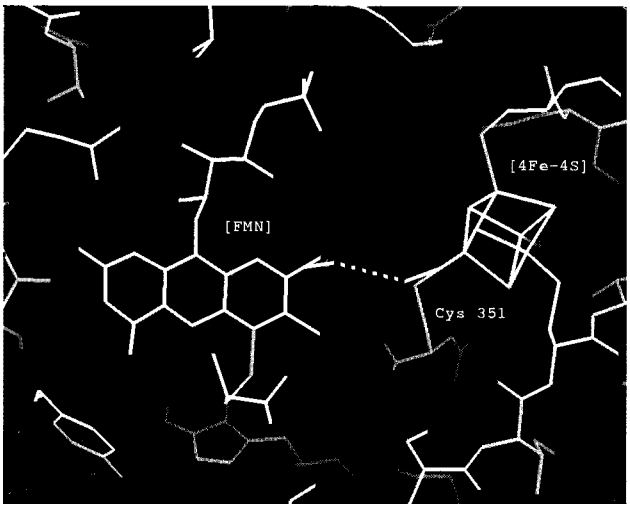


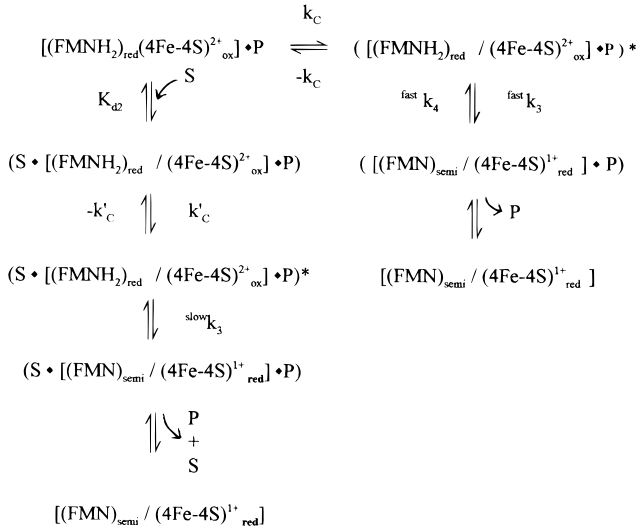
FIGURE 4: Possible pathway for intramolecular ET in TMADH. This figure was generated using the coordinates of the known crystal structure (Lim et al., 1986; Barber et al., 1992). Both the FMN and iron−sulfur centers are labeled, as well as the cysteine residue 351 which is involved in all of the most efficient pathways predicted by the Greenpath program. The shortest ET pathway includes a 2.91 Å through-space jump (dashed line) from one of the hydrogens on the 8α-methyl group of FMN to the lone pair orbital of the cysteine^{351} sulfur.

describe the two alternative ET reactions are the same, then the large H_{AB} value associated with $^{fast}k_3$ must correspond to a shorter ET distance (discussed later).

Ionic Strength Dependence. One would intuitively expect that a true ET reaction should be ionic strength independent. However, there are reports of ionic strength dependencies of ET rates within protein complexes measured by transient kinetic techniques. The ionic strength dependence could be a consequence of ET being coupled to some structural or electronic changes within the enzyme (Hazzard et al., 1991; Harris et al., 1994; Brunschwig & Sutin, 1989). To investigate this possibility, the rate of intramolecular ET in TMADH was measured in 10 mM potassium phosphate buffer, pH 7.5 at 30 °C (Figure 3). Under these low ionic strength conditions $^{fast}k_3$, which was approximately 150 s^{-1} in 0.1 M buffer, was now too rapid to be measured accurately ($^{fast}k_3 > 500\ s^{-1}$). Also, the k_4 value at the low ionic strength was essentially zero, suggesting that the equilibrium constant for faster ET reaction is dependent on ionic strength. In contrast, the limiting value of $^{slow}k_3$ was 5.7 ± 1, which is essentially the same as what was measured in 0.1 M buffer. The reaction was also repeated at 30 °C in 0.1 M potassium phosphate buffer, with 0.2 M NaCl present (data not shown). The k_{obs} for each phase again showed a hyperbolic concentration dependence with respect to trimethylamine. The value of $^{fast}k_3$ again showed a dependence on ionic strength and decreased to 60 ± 4 s^{-1}. The value of $^{slow}k_3$ was again relatively unaffected and was determined to be 5 ± 2 s^{-1}.

Pathways Analysis of the TMADH Structure. A Pathways analysis (Regan et al., 1993) of TMADH was performed using the Greenpath v0.97 computer program. Several pathways which were within 50% efficiency of the best pathway were identified. All of the dominant pathways used essentially the same route from FMN to the iron−sulfur center, irrespective of where the electron left the FMN moiety and where it was directed to go on the iron−sulfur center. A representation of the relevant portion of TMADH is illustrated in Figure 4. Both FMN and the iron−sulfur

Scheme 1



centers are labeled as well as the cysteine residue 351, a ligand of the iron−sulfur center which is involved in all of the pathways. The shortest direct distance from an iron atom to the isoalloxazine ring of FMN is 7.0 Å from the FE3 iron to the C8 carbon of the FMN ring. Depicted in this diagram is the shortest ET pathway calculated between the two cofactors. In all of the dominant pathways the electron always exited from one of the C−H bonds of the 8α-methyl group, jumped through space a distance of 2.91 Å to the lone pair orbital of the sulfur of Cys^{351} which is bonded to the iron−sulfur center, and entered the center via the FE3 iron. All of the dominant pathways contained only this one through-space jump. Significantly less efficient pathways were also predicted in which the electron exited from the 7α-methyl group, which required a longer 3.25 Å jump through space to Cys^{351}.

DISCUSSION

We previously presented a model to describe the biphasic intramolecular ET reactions which occur subsequent to the reduction of FMN in TMADH by substrate (Falzon & Davidson, 1996). An expanded version of this model is given in Scheme 1 to account for the possibility that each of the two alternative intramolecular ET events (depicted here as $^{slow}k_3$ and $^{fast}k_3$) may be preceded by a non-ET event such as a conformational change. In this scheme P represents the dimethylamine and formaldehyde products, while S represents the second trimethylamine molecule which binds to reduced TMADH. The rate constants k_C and k_C' describe conformational reorientations or catalytic events which are required to achieve the activated form of the protein molecule in which the ET occurs for the fast and slow rates, respectively. The points at which P and S are released are not known and may be different than are shown here. The star (*) signifies an unstable conformer of the enzyme in which ET occurs, and it differs from its stable counterpart in that it might have a different nuclear configuration or electronic structure. The spectra of the stable and unstable conformers are assumed to be similar so that their interconversion cannot be observed directly.

We have previously shown that examination of the temperature dependence of an apparent ET rate constant can provide insight into whether or not that ET reaction is gated

by or coupled to a non-ET event (Bishop & Davidson, 1995). Analysis of the temperature dependence of $^{slow}k_3$ and $^{fast}k_3$ yielded values of H_{AB} which were within the nonadiabatic limit (Table 2). This suggests that for both the slow and fast ET reactions the ET events are truly rate-limiting. As discussed earlier, in a nonadiabatic process achievement of the transition-state energy does not always lead to product formation. The probability that the achievement of the transition-state energy leads to the formation of product is less than unity and approaches zero as H_{AB} approaches zero. If the actual ET step is truly rate-limiting for the experimentally determined k_{ET} then H_{AB} should at least be less than 80 cm^{-1}. This limit is calculated from the dynamic relaxation rate of water and occurs when the reorganization of water, as the bulk solvent, becomes rate-limiting (Winkler & Gray, 1992). Because nonadiabatic ET reactions occur within an ET complex, they are collisionless. So in this case interpretation of the parameters calculated from transition-state theory is not clear as such reactions do not involve formation or breakage of bonds. For such reactions the standard activation entropy and enthalpy changes are better described by λ and H_{AB}.

Analysis of the temperature dependence of the apparent ET rates by eq 3 indicates that $^{slow}k_3$ and $^{fast}k_3$ exhibit significantly different values of H_{AB}. It is important to note that the experimentally determined ET distances calculated from eq 4 (Table 2) are in good agreement with the crystal structure. The direct center to center distance from the flavin cofactor to the iron−sulfur cluster in the crystal structure of the enzyme is about 12 Å, and the 8α-carbon of the FMN is only about 7 Å from the closest iron atom. The length of the shortest pathway from the FMN ring to the iron−sulfur center is about 8 Å. Assuming a β value intermediate between 0.7 and 1.4 $Å^{-1}$, the predicted distances between the two redox centers based on our experimental data were 8.1 Å for the fast phase and 12.3 Å for the slow phase. It is feasible that the value of H_{AB} could be altered by a conformational change, even a relatively subtle perturbation that could change the ET distance between redox centers or change the length of a critical through-space jump such that a completely different pathway becomes preferable. The observed differences in H_{AB} could be the result of a small conformational change which is induced by the binding of the second molecule of substrate. Alternatively, that second binding event may cause the electron distribution on the FMN ring to change such that the ET distance to the iron−sulfur center is changed. A comparison of the range of distances predicted by eq 4 for $^{slow}k_3$ and $^{fast}k_3$ indicates that the binding of the second substrate molecule to reduced TMADH leads to a change within the enzyme, such that the ET pathway becomes longer or less efficient. The binding-induced event which leads to this change might be subtle enough that it would be difficult to detect.[2]

It is of particular interest to mention the unusual EPR spectrum of the substrate-reduced TMADH. It shows a complex pattern of signals including an unusual half-field $g = 4$ signal (referred to as the triplet state) indicative of spin-coupling between two paramagnetic species, presumably the flavosemiquinone and the reduced iron−sulfur center (Steenkamp et al., 1978a; Steenkamp & Beinert, 1982a). Recently, Bertrand et al. (1995) proposed that the absence of magnetic interaction between the FMN cofactor and the [2Fe-2S] center in phthalate dioxygenase reductase is due to the existence of "magic" configurations of the two centers for which the dipolar terms cancel. In the case of a $[2Fe\text{-}2S]^+$ cluster interacting with a mononuclear center or radical, it occurs when the angle between the vectors of the center of the $[2Fe\text{-}2S]^+$ and the center of the mononuclear center is close to 135° (Bertrand et al., 1995). What this indicates is that the angle between the centers of the two cofactors is crucial for the magnetic interaction of the two species. Thus, formation of the triplet state may not necessarily require gross geometrical rearrangement of the enzyme molecule, but subtle tilting of one cofactor relative to the other may be sufficient to cause the two centers to interact and form the triplet state. In view of these findings, one may consider the possibility that the differences in H_{AB} values for $^{slow}k_3$ and $^{fast}k_3$, and also the slower rates of ET in the presence substrate relative to those measured in the absence of substrate by pH-jump (Rohlfs & Hille, 1991), may be related to differences in coupling between the two redox centers caused by subtle changes in their orientations. The suggestion that some geometrical reorientation of the two cofactors relative to each other is required to optimize the ET event may also help to explain the relatively large experimentally determined λ values for the slower and faster intramolecular ET reactions.

The λ value of 2.2 eV reported here for both the slow and fast rates is relatively large for a true intramolecular ET reaction (Winkler & Gray, 1992). This may be a consequence of a required change in enzyme conformation (or other types of configurational, structural, or electronic changes) for an optimal ET reaction. When ET is rate-limiting for an observed ET reaction, but that rate is attenuated by some adiabatic prerequisite reaction step, the reaction may be thought of as a coupled ET reaction (Brunschwig & Sutin, 1989; Harris et al., 1994). A conformationally coupled ET reaction is one in which the observed ET rate constant is controlled by a fast but unfavorable conformational change that precedes the ET step. The experimentally determined rate constant is equal to the product of the true ET rate constant and the equilibrium constant for the conformational change (eq 7). Furthermore,

$$k_3 = k_{ET}K_C \tag{7}$$

the value for the experimentally determined reorganizational energy, λ_{obs}, will contain contributions from the actual ET step, λ_{ET}, as well as the conformational preequilibrium step, λ_C, so that $\lambda_{obs} = f(\lambda_{ET},\lambda_C)$. As discussed in Bishop and

[2] Crystallographic studies (Bellamy et al., 1989) have shown that there are no gross changes in conformation when the substrate analog TMAC is bound to TMADH. However, very subtle perturbations of structure may significantly affect ET. According to the Pathways algorithm (Regan et al., 1993) the decay factor (ϵ) for a through-space jump is proportional to $e^{-(1.7(r-1.4))}$ where r is the distance of the jump and 1.4 Å is used as a reference covalent bond distance. H_{AB} is a product of all ϵ values associated with the pathway, and k_{ET} is proportional to H_{AB}^2 (see eq 4). For the pathway in Figure 4, one may calculate that increasing the distance of the 2.9 Å through-space jump by only 0.7 Å would result in a greater than 10-fold decrease in k_{ET}. Thus very subtle changes in the orientations of the cofactors or electron distribution within the cofactors may have a dramatic effect on k_{ET}.

Davidson (1995) this prerequisite step need not be a conformational change but could also be a catalytic event such as protonation/deprotonation. It should also be noted that the apparent ET rate measured in the absence of substrate in the pH-jump experiments (Rohlfs & Hille, 1991; Rohlfs et al., 1995) is much faster than the apparent ET rates in our study. This may also be a consequence of the substrate-dependent intramolecular ET reactions being coupled (see eq 7).

A role for conformational reorientations may intuitively seem less relevant for an intramolecular ET reaction than an interprotein reaction. Recent studies on the hydrodynamic properties of TMADH (Colfen et al., 1996), however, strengthen the notion that they may be important for this enzyme. It was reported that TMADH is conformationally flexible and contains significantly more internal solvent in solution than in the crystalline state, nearly a doubling of effective volume, which is more than is typically observed or expected. It must be noted that unlike many model protein ET systems (e.g., blue copper proteins, *c*-type cytochromes), TMADH is a large, dimeric, globular protein. For such a protein, dynamic fluctuations in conformation certainly occur. Some of these may be required to optimize coupling for ET and be reflected in λ_{obs}.

The ionic strength dependence of $^{fast}k_3$ is interesting and may be consistent with that reaction being coupled ET. There are a number of reports of the ionic strength dependencies of ET rates within protein complexes measured by transient kinetic techniques. The rate constant for the ET reaction between methanol dehydrogenase and cytochrome *c*-551i increased 2-fold with increasing ionic strength (μ) from 0.21 to 1.3 M (Harris et al., 1994). A decrease in the ET rate constant from 85 s^{-1} at $\mu = 48$ mM to 66 s^{-1} at $\mu = 76$ mM was reported for flavodoxin−cytochrome *c* complex (Simondsen et al., 1982). A decrease in the rate constant from 4000 s^{-1} at $\mu = 310$ mM to 1600 s^{-1} at $\mu = 460$ mM was reported for the ferredoxin−ferrodoxin−$NADP^+$ reductase complex (Bhattacharrya et al., 1986). Harris et al., (1994) explain their results as being due to the coupling of a highly unfavorable conformational change that precedes the actual ET step. Similarly, the other previously reported ionic strength dependencies of observed ET rate constants have also been attributed to the formation of nonoptimal complexes that require geometrical rearrangement prior to the ET. A similar argument has also been made to rationalize the "anomalous" rate of intramolecular ET between iron and ruthenium centers in a modified cytochrome *c* (Bechtold et al., 1986). The oxidation of the Fe(II) heme by $Ru^{III}(NH_3)_4$-(isn) attached to the histidine-33 residue of cytochrome *c* is much slower than the reduction of the Fe(III) heme by a bound $Ru^{II}(NH_3)_5$ moiety despite the very similar driving forces for the two reactions. This apparent dependence of the rate on direction was explained by proposing that the Fe(II) protein undergoes a conformation change prior to its oxidation to Fe(III).

If $^{fast}k_3$ for the intramolecular ET between FMN and iron−sulfur center in TMADH contains a component that reflects such a conformational rearrangement, the equilibrium constant of which is dependent on ionic strength, this could explain why $^{fast}k_3$ is dependent on ionic strength. The large apparent λ_{obs} of 2.2 eV for this reaction would then likely be a consequence of the contribution from λ_C. Similarly, the same argument can be applied to explain the large λ_{obs} of 2.2 eV obtained for the $^{slow}k_3$. However, no ionic strength dependence of the slow rate was observed. This seems to indicate that the process coupled to the slower reaction is different than the process coupled to the faster reaction, and the similar values of λ_{obs} may be coincidental. The differences in the ionic strength dependence of the two rates do confirm that $^{slow}k_3$ and $^{fast}k_3$ describe different alternative intramolecular ET reactions. The absence of an ionic strength dependence of $^{slow}k_3$ indicates that the observed effect on $^{fast}k_3$ is not simply an effect of ionic strength on the redox potentials of the cofactors. If it were, an effect on both rates would have been observed. How the reactions described by $^{slow}k_3$ and $^{fast}k_3$ differ mechanistically cannot at this time be determined.

Although values of λ which are obtained from the temperature dependence of k_{ET} may be subject to experimental artifacts, we believe the values reported here are valid. A complication which may arise is the possible temperature dependence of $\Delta G°$. This could be due to temperature-dependent conformational changes in TMADH which may affect the redox potential of either or both cofactors. TMADH is thermally stable over the range of temperatures used in this study, which suggests that at least no gross conformational changes are occurring in the protein over this temperature range. Also, for $^{fast}k_3$ the kinetically-determined $\Delta G°$ (calculated from the ratio of k_3/k_4) does not vary appreciably over this temperature range and is consistent with the known redox potentials of the cofactors. It should also be noted that for these reactions the value of λ is very large (2.2 eV) relative to $\Delta G°$ (-6.4 kJ $mol^{-1} = 0.066$ V). Thus, any changes in $\Delta G°$ would have to be enormous to compromise the analysis by eqs 3 and 4, and this seems very unlikely.

Rohlfs et al. (1995) have recently investigated the kinetics of the interconversion of the FMN hydroquinone, FMN semiquinone and the spin-interacting triplet state in TMADH using a pH-jump technique in both H_2O and D_2O. The observed kinetics were consistent with a reaction mechanism linking sequential protonation/deprotonation and intramolecular ET events. If the reactions studied in this paper were gated by proton transfer one would not expect the results discussed above which led us to the conclusion that for both $^{slow}k_3$ and $^{fast}k_3$ the intramolecular ET steps are rate-limiting. However, care must be taken when comparing studies performed under different reaction conditions, and certain issues must be considered. Rohlfs et al. used dithionite-reduced TMADH, whereas the substrate-reduced enzyme was used in the present study. It is known that pronounced differences exist in the kinetics of dithionite- and substrate-reduced TMADH. The observed rates for the dithionite-reduced TMADH (Rohlfs & Hille, 1991; Rohlfs et al., 1995) are 10−100-fold larger than the rates of intramolecular ET reported for substrate-reduced enzyme (Steenkamp et al., 1978a,b; Rohlfs & Hille, 1994; Falzon & Davidson, 1996). In addition, EPR spectra of TMADH reduced to the level of two equivalents by dithionite at pH values of less than 10 showed that, unlike in substrate-reduced TMADH, the flavin semiquinone and reduced iron−sulfur center are not ferromagnetically coupled. Perhaps most significantly, in the experiments with dithionite-reduced TMADH the intramolecular ET was initiated by a pH-jump technique, while in this study which was carried at physiologic pH, ET was initiated by the addition of substrate to the oxidized enzyme.

Rohlfs et al. (1995) conclude that deprotonation of $FMNH_2$ to $FMNH^-$ must precede intramolecular ET. The same may be true for the ET reactions from substrate-reduced TMADH at pH 7.5 (our current results). It may be that when the ET reaction of dithionite-reduced TMADH is initiated by pH-jump the rate of deprotonation is slower than the actual ET rate and so the reaction is gated by this step. That rate is still significantly faster than the rates which we measure under our experimental conditions. That may mean that under our conditions the rate of the actual ET step is now slower than the rate of deprotonation so that the observed reaction is no longer gated by that step. Whether or not a reaction is gated depends on the relative rates of the ET and prerequisite non-ET steps. The combined results of these studies underscore the complexity of the reactions which occur in this relatively simple metalloflavoprotein. Further studies will be needed to answer the important question of how the binding of one or more substrate or product molecules so dramatically influences the ET properties of TMADH.

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