Kinetic Model for the Regulation by Substrate of Intramolecular Electron Transfer in Trimethylamine Dehydrogenase†

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ABSTRACT: The reaction of trimethylamine dehydrogenase (TMADH) with trimethylamine has been studied by rapid-scanning stopped-flow spectroscopy and steady-state kinetics. The covalently bound 6-*S*-cysteinylflavin mononucleotide (FMN) cofactor is initially reduced by substrate and exhibits a limiting first order rate constant of 230 s^{-1} at pH 7.5 and 30 °C. One electron is then transferred intramolecularly from the reduced $FMNH_2$ to the oxidized $[4Fe\text{-}4S]^{2+}$ center. This reaction is biphasic, and the extent of the reaction which corresponds to the faster and slower rates is dependent upon the concentration of trimethylamine. The limiting first order rate constants are 160 and 4 s^{-1}. At low substrate concentrations, the faster rate is dominant, and at high substrate concentrations, the slower rate is dominant. These results are used to develop a model for the reductive half-reaction of TMADH in which two molecules of substrate bind to TMADH. One binds at the active site of oxidized TMADH and is converted to products. A second molecule binds but is not converted to products and influences the rate of intramolecular electron transfer. Analysis of the transient kinetic data yielded apparent dissociation constants for trimethylamine of 36 and 148 μM, respectively, for binding to the catalytic and noncatalytic sites. Steady-state kinetic studies indicated substrate inhibition which was best described by a model in which binding of a second molecule of trimethylamine causes a 10-fold reduction in k_{cat} from 11 to 1.1 s^{-1}. This suggests that, at high substrate concentrations, the rate of the intramolecular electron transfer reaction has become sufficiently slow to be at least partially rate-limiting for the steady-state reaction. These kinetic data are interpreted in the context of the known crystal structure of TMADH. The mechanistic implications regarding long range electron transfer and possible physiologic significance of these findings are discussed.

Trimethylamine dehydrogenase (TMADH),[1] an enzyme from the restricted facultative methylotrophic bacterium *Methylophilus methylotrophus* sp. W3A1, catalyzes the oxidative N-demethylation of trimethylamine to dimethylamine and formaldehyde (Colby & Zatman, 1974). The structure of TMADH has 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). 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 [4Fe-4S] cluster (Hill et al., 1977), which are involved in catalysis and the subsequent electron transfer to its physiological electron acceptor, electron transfer flavoprotein (ETF) (Steenkamp & Gallup, 1978; Davidson et al., 1986). As such, TMADH may be considered one of the simplest and structurally best characterized examples of a complex metalloflavoprotein and an ideal physiologic system in which to study the mechanisms of long range electron transfer through proteins.

Previous studies have indicated that the interaction between the FMN and [4Fe-4S] centers of TMADH is profoundly influenced by the presence of substrate or substrate analogs (Steenkamp et al., 1978a; Steenkamp & Beinert, 1982a; Beinert et al., 1982). Pronounced differences have been observed between dithionite- and substrate-reduced TMADH, both by EPR and optical spectroscopy. When TMADH is reduced chemically by dithionite, three electrons are taken up per subunit of enzyme, while when reduced by substrate, only two electrons are taken up. The EPR spectrum of the substrate-reduced enzyme was of particular interest since it showed 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 cluster (Steenkamp et al., 1978a; Steenkamp & Beinert, 1982a). No indication of the occurrence of this spin-coupled interacting form was observed during titration with dithionite, suggesting that the presence of bound substrate or product somehow affects the relative orientations of the two redox centers within TMADH (Steenkamp et al., 1978a). The idea that trimethylamine binding enhances the formation of the triplet state was confirmed with tetramethylammonium chloride (TMAC), which is a substrate analog and inhibitor of TMADH. When a dithionite titration was performed in the presence of TMAC, only two electrons were taken up by TMADH and the triplet state was formed, similar to what was observed upon reduction by substrate (Steenkamp et al., 1978a; Steenkamp & Beinert, 1982a). Interestingly, full development of this unusual EPR spectrum required more than 1 mol of trimethylamine per mole of enzyme subunit,

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[1] Abbreviations: TMADH, trimethylamine dehydrogenase; TMAC, tetramethylammonium chloride; FMN, flavin mononucleotide; k_{ET}, electron transfer rate constant; PES, phenazine ethosulfate; PMS, phenazine methosulfate; ETF, electron transfer flavoprotein; EPR, electron paramagnetic resonance.

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suggesting the presence of a second noncatalytic, and possibly allosteric, binding site. The existence of such a second binding site for substrate was demonstrated by gel chromatography after binding of [^{14}C]trimethylamine to reduced TMADH (Steenkamp & Beinert, 1982a). The amount of additional [^{14}C]trimethylamine bound to reduced TMADH was determined to be 0.87 mol per mole of subunit.

Steady-state kinetic studies of TMADH using phenazine methosulfate (PMS) as an artificial electron acceptor have shown TMADH to react via a ping-pong mechanism (Steenkamp & Mallison, 1976). However, steady-state kinetic data on the reactions of TMADH have not been readily interpretable. It was initially reported that product inhibition accounted for unusual steady-state kinetic behavior of the enzyme, and a mechanism was proposed which included conformational isomerization of enzyme forms (Steenkamp & Mallison, 1976). It was subsequently reported that TMADH exhibited partial substrate inhibition at high substrate concentrations (Steenkamp & Beinert, 1982b). It is however clear that product release must precede binding of the electron acceptor. Product inhibition patterns with the alternative substrate, diethylamine, indicated that the release of the ethylamine product occurred before addition of PMS (Steenkamp & Mallison, 1978). Also, after reduction of TMADH with [^{14}C]trimethylamine and chromatography in the absence of a reoxidant, essentially no label was retained (Steenkamp et al., 1978a). It is important to note that this substrate inhibition is not an artifact observed only with artificial electron acceptors. In steady-state studies using ETF as an electron acceptor, the reduction rate at a fixed concentration of ETF and at varying concentrations of trimethylamine showed a marked substrate inhibition in the millimolar range (Steenkamp & Gallup, 1978), similar to what was observed with PMS.

Previous stopped-flow spectrophotometric studies of the reductive half-reaction of TMADH have revealed multiple kinetic phases. Kinetic studies have shown that the FMN initially accepts two electrons from the substrate and then one electron is transferred intramolecularly from $FMNH_2$ to $[4Fe\text{-}4S]^{2+}$ (Steenkamp & Beinert, 1982b). 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,c; Bellamy et al., 1989; Rohlfs & Hille, 1994). The relative slowness of the rate of intramolecular electron transfer from $FMNH_2$ to $[4Fe\text{-}4S]^{2+}$ is surprising given the close proximity of the two redox centers. The crystal structure of TMADH indicates that the 8α-methyl 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 $FMNH_2$ in the absence of substrate is significantly faster, suggesting that bound substrate or product somehow reduces the observed rate of electron transfer from $FMNH_2$. This suggests that this intramolecular electron transfer reaction may be gated (Hoffman & Ratner, 1987) by a slower process or coupled (Harris et al., 1994) to some other highly unfavorable process.

This intramolecular electron transfer reaction is physiologically important because ETF, the natural electron acceptor for TMADH, only accepts a single electron. ETF from bacterium *M. methylotrophus* W3A1 is composed of two dissimilar subunits (M_r = 42 000 and 38 000) and contains 1 mol of flavin adenine dinucleotide (FAD) per mole of protein (Steenkamp & Gallup, 1978; Davidson et al., 1986). Although ETF has a two-electron capacity it is only reduced to the level of semiquinone which resists further reduction (Davidson et al., 1986). Full reduction could only be achieved by electrochemical means (Byron et al., 1989). These data indicate that the anionic semiquinone form of this ETF is an extremely stable intermediate and suggests that in vivo *M. methylotrophus* ETF transfers one electron at a time, cycling between the fully oxidized and radical forms.

In this paper, we have reexamined the steady-state and transient kinetic properties of TMADH. These kinetic data are used to propose a model 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. As previously noted, it is required for full development of the triplet state of the substrate-reduced enzyme. In this paper, we characterize the manner in which it inhibits the steady-state turnover of TMADH and demonstrate that it may act as a switch between alternative mechanisms that regulate the rate of the intramolecular electron transfer reaction from $FMNH_2$ to $[4Fe\text{-}4S]^{2+}$. The mechanistic implications and possible physiologic significance of these findings are discussed.

EXPERIMENTAL PROCEDURES

M. methylotrophus sp. W3A1 (NCIB 11348) was grown on the minimal medium of Owens and Keddie (1969) using 0.5% dimethylamine as a carbon source. Dimethylamine rather than trimethylamine was used as a carbon source to minimize the amount of slime produced by this bacterium during growth (Davidson, 1985). TMADH was purified as described by Steenkamp and Mallison (1976) with certain modifications as follows. After sonication of the cells and centrifugation at 17700*g* for 30 min, the supernatant was collected and recentrifuged twice at 17700*g* for 30 min. The additional centrifugation steps were performed to get rid of any slime still retained in the supernatant. This facilitated the subsequent purification steps. Gel filtration of the cell extract was performed using a Spectrum AcA34 gel filtration column rather than Sephadex G-200. 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).

Stopped-flow experiments were performed 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 M potassium phosphate buffer (pH 7.5) at 30 °C. The rates of formation of $FMNH_2$ (represented by the change in absorbance at 443 nm) and the subsequent one-electron oxidation to form the flavinsemiquinone (represented by the change in absorbance at 365 nm) were slow enough under these conditions to be measured by stopped-flow spectroscopy (Figure 1). The spectrum which is characteristic of each redox form is known from previous freeze−quench EPR

and absorbance spectroscopy studies (Steenkamp & Beinert, 1982a; Beinert et al., 1982; Steenkamp et al., 1978a). For each trimethylamine concentration, at least four replicas, each containing 1000 data points, were collected, fitted to the appropriate equation for an exponential rise or fall, and averaged. Under these pseudo-first order conditions, the observed rate constant (k_{obs}) for the absorbance change at 443 nm was best obtained from fits of the data to the equation for a single exponential decay (eq 1), where C is a constant

$$A_{443} = Ce^{-kt} + b \quad (1)$$

related to the initial absorbance and b represents an offset value to account for a non-zero base line. In contrast, the data obtained at 365 nm were biphasic and best described by eq 2.

$$A_{365} = C_1(1 - e^{-k^{fast}t}) + C_2(1 - e^{-k^{slow}t}) + b \quad (2)$$

where k^{fast} and k^{slow} are the observed rate constants for the faster and slower rates, respectively. C_1 and C_2 are related to the initial absorbance, and b is an offset value to a account for a non-zero baseline.

Microscopic rate constants were determined from analysis of the dependence of k_{obs} on the concentration of the varied reactant. For each reaction which was studied, k_{obs} was described by simple models of the form given in eq 3. The

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C \underset{k_4}{\overset{k_3}{\rightleftharpoons}} D \quad (3)$$

species which are represented by A−D for each reaction are described in the results. Given this model, the concentration dependence of k_{obs} was fit to either eq 4 or 5, which are

$$k_{obs} = \frac{k_3[S]}{K_d + [S]} + k_4 \quad (4)$$

$$k_{obs} = 0.5\{k_1[S] + k_2 + k_3 + k_4 - [(k_1[S] + k_2 + k_3 + k_4)^2 - 4k_1k_3[S] - 4k_1k_4[S] - 4k_2k_4]^{0.5}\} \quad (5)$$

derived, respectively, in Strickland et al. (1975) and Hiromi (1979). Use of eq 4 is appropriate if $k_1[S] + k_2 \gg k_3 + k_4$. When this condition is met, the variation in k_{obs} with [S] will be described by a simple hyperbola. If one attempts to fit such data to eq 5, it will not be possible to obtain unique values for k_1 and k_2 . A unique value may only be obtained for the ratio k_2/k_1 . When this condition is not met, then eq 5 must be used. Attempts to fit data to eq 4 would display a systematic deviation from the fit because the plot of k_{obs} versus [S] will not be hyperbolic. Nonlinear curve fitting of these data was performed with the Sigma Plot 5.1 (Jandel Scientific, San Rafael, CA) computer program.

Steady-state kinetic measurements were performed spectrophotometrically, essentially as described by Colby and Zatman (1974), with a Uvikon 810 spectrophotometer using a dye-linked assay which employs oxidized phenazine ethosulfate (PES) as an electron acceptor. The reduction of TMADH is coupled by PES to a change in the absorbance of a redox-sensitive dye, 2,6-dichlorophenolindophenol (ϵ = 21 500 M^{-1} cm^{-1}). The assay mixture contained 20 nM TMADH in 0.1 M potassium phosphate buffer (pH 7.5) at 30 °C, and each reaction was initiated by the addition of trimethylamine.

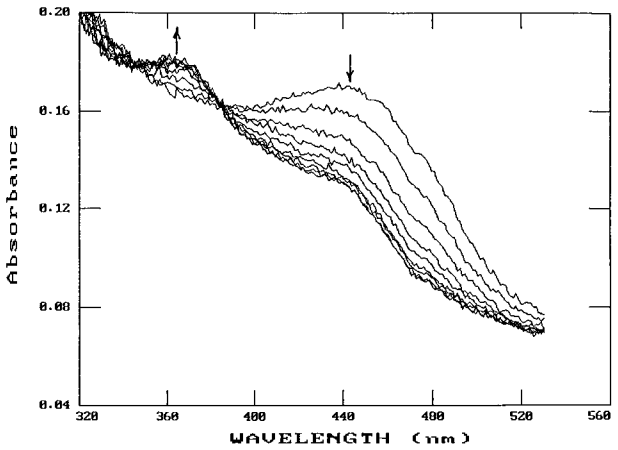


FIGURE 1: Reaction of TMADH with trimethylamine. TMADH (2 μM) was mixed with trimethylamine (200 μM) in 100 mM potassium phosphate buffer (pH 7.5) at 30 °C. Arrows indicate the direction of the spectral change with time. Spectra were recorded at 8 ms intervals.

RESULTS

Stopped-Flow Studies of the Reductive Half-Reaction. The reaction of TMADH with trimethylamine was examined by rapid-scanning stopped-flow spectroscopy (Figure 1). With our instrumentation, it is possible to record an entire spectrum of the region of interest once every millisecond. This has allowed us to characterize two discrete steps in the reductive half-reaction of TMADH (eq 6), where S and P are,

$$S + FMN/[4Fe\text{-}4S]^{2+} \rightarrow P + FMNH_2/[4Fe\text{-}4S]^{2+} \rightarrow P + FMN_{semi}/[4Fe\text{-}4S]^{+} \quad (6)$$

respectively, substrate and products. The first step is the substrate-dependent two-electron reduction of FMN to $FMNH_2$. The second step is the subsequent intramolecular one-electron transfer from $FMNH_2$ to $[4Fe\text{-}4S]^{2+}$. The point in the reaction coordinate at which time product release occurs is not known. These two reactions may be monitored independently at 443 and 365 nm, respectively. The spectrum which is characteristic of each redox form is known from previous freeze−quench EPR and absorbance spectroscopy studies (Steenkamp & Beinert, 1982a; Beinert et al., 1982; Steenkamp et al., 1978a).

The absorbance change with time at 443 nm (representing flavin reduction), which was observed upon mixing TMADH and trimethylamine, followed a single exponential decay (eq 1, Figure 2A), suggesting a direct two-electron reduction of FMN by substrate with no involvement of the semiquinone. To obtain rate constants for this reaction, values of k_{obs} were obtained at several different concentrations of trimethylamine. Using the model in eq 7, these data were fit to eq 5

$$S + FMN/[4Fe\text{-}4S]^{2+} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} S^*FMN/[4Fe\text{-}4S]^{2+} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} P^*FMNH_2/[4Fe\text{-}4S]^{2+} \quad (7)$$

(Figure 2B).[2] Attempts to fit these data to eq 4 yielded unacceptable results. A systematic deviation from the fit

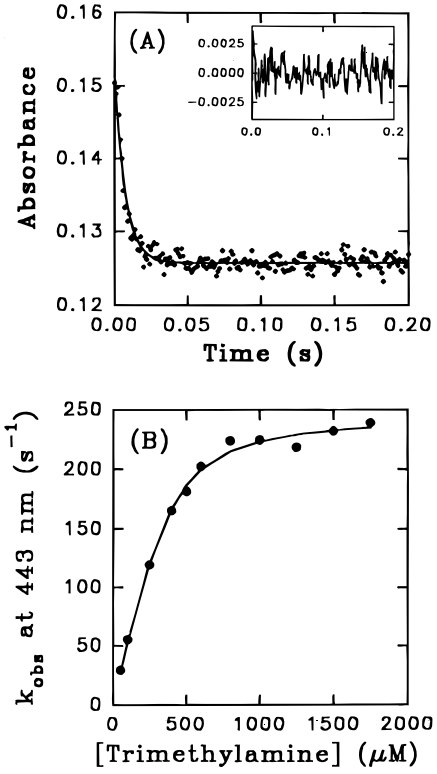


FIGURE 2: (A) Reduction of the FMN cofactor of TMADH by trimethylamine as monitored by the decrease in absorbance at 443 nm. TMADH (3 μM) was mixed with trimethylamine (800 μM) in 100 mM potassium phosphate buffer (pH 7.5) at 30 °C. The solid line represents the fit of these data to eq 1, which for this data set gave a value for k_{obs} of 147 ± 4 s^{-1}. The inset shows a plot of the residuals for this fit. (B) Plot of k_{obs} against substrate concentration. The values of k_{obs} were determined at several concentrations of substrate. The solid line represents the fit of these data to eq 5.

was observed, indicating that this alternative model was inappropriate. The fit to eq 5 yielded a k_4 of approximately zero, but very large errors for the fitted values of k_1, k_2, and k_3. To minimize the errors, the data were refit to eq 5 with k_4 set to zero to obtain rate constants of $k_3 = 232 \pm 9$ s^{-1}, $k_1 = 0.5 \pm 0.07$ s^{-1} μM^{-1} and $k_2 = 18 \pm 14$ s^{-1} ($K_d = 36 \pm 28$ μM). It had been reported previously that the rate of FMN reduction in TMADH by trimethylamine was too fast to be studied by stopped-flow spectroscopy, particularly at high concentrations of trimethylamine and at high pH (Steenkamp et al., 1978c; Rohlfs & Hille, 1994). However, this rate is readily measurable under our experimental conditions. To be certain that our TMADH preparation was comparable to that used by others, the rate of reaction of TMADH was also measured with diethylmethylamine, a slow-reacting alternative substrate. The rates which we obtained were nearly identical to those reported by Rohlfs and Hille (1994) with that substrate under similar experimental conditions (data not shown).

[2] Use of eq 5 indicates that the time-dependent concentration of P*FMNH$_2$/[4Fe-4S]$^{2+}$ should equal k_1k_3[E][S]{$1/\alpha\beta + e^{-\alpha t}/[\alpha(\alpha - \beta)] + e^{-\beta t}/[\beta(\beta - \alpha)]$}, where α and $\beta = k_{obs}$ as defined in eq 5 (Capellos & Bielski, 1972). The primary data, such as is shown in Figure 2A, can also be fit well to the appropriate form of this equation. Examination of the plots of the residuals to these fits reveals that the plots are indistinguishable from those obtained from fits to eq 1. Use of this more complex equation does not improve on the quality of the fit of the data obtained from use of eq 1. Thus, the simplifying assumptions implicit in the use of eq 1 to analyze the raw data are valid, and these fits provide reasonable estimates of k_{obs}.

The change in absorbance with time at 365 nm, representing the one-electron oxidation of the reduced flavin by the oxidized [4Fe-4S]$^{2+}$ center, best fit to a two-exponential equation (eq 2, Figure 3A). The biphasic behavior at this wavelength was observed throughout the range of trimethylamine concentrations used. The two observed rate constants were each analyzed independently. The microscopic rate constants which describe each observed rate were determined by examination of each k_{obs} as a function of substrate concentration, and using for each the simple model given in eq 8. Thus, it was initially assumed that the biphasic

$$S + FMN/[4Fe\text{-}4S]^{2+} \overset{K_d}{\rightleftharpoons} P^*FMNH_2/[4Fe\text{-}4S]^{2+} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} P^*FMN_{semi}/[4Fe\text{-}4S]^{+} \quad (8)$$

behavior represented two alternative mechanisms by which the formation of the flavinsemiquinone occurs. 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 8. These data sets could each be described by a simple hyperbola and, for the reasons discussed earlier, were fit to eq 4 (Figure 3B,C). Values were obtained for k_3 and K_d for both phases. For the faster phase, $k_3 = 160 \pm 19$ s^{-1} and $K_d = 632 \pm 20$ μM. For the slower phase, $k_3 = 4 \pm 0.6$ s^{-1} and $K_d = 608 \pm 21$ μM. Values of k_4 for both phases approximated zero, which is consistent with the difference in redox potentials between the FMNH$_2$/FMN$_{semi}$ couple of 36 mV and the [4Fe-4S]$^{+2}$/[4Fe-4S]$^{+}$ couple of 102 mV (Barber et al., 1988). The potential difference of 66 mV predicts that the electron transfer from FMNH$_2$ to [4Fe-4S]$^{2+}$ will be nearly irreversible. These kinetically determined K_d values are obviously not true K_ds (see eq 8). 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-4S]$^{2+}$. The true significance of these K_d values will require additional studies and ultimate derivation of a detailed kinetic model for the reductive half-reaction. It is, however, noteworthy that the apparent K_ds which were obtained for each of the two phases are essentially identical. This suggests that the events which precede the faster and slower intramolecular electron transfer reactions may be identical.

Each limiting first order rate constant (k_3) for the redox reaction may be described as an apparent electron transfer rate constant (k_{ET}). These data suggest that the intramolecular electron transfer from FMNH$_2$ to [4Fe-4S]$^{+2}$ is occurring by two alternative processes, each of which exhibits a different rate. While each k_3 describes the rate of an electron transfer (redox) reaction, the slowness of the rates (discussed earlier) and the fact that different rates are observed for the same reaction suggest that each of these alternative electron transfer reactions may be either gated (Hoffman & Ratner, 1987) by a slower process or coupled (Harris et al., 1994) to some other highly unfavorable process. Furthermore, the non-electron transfer processes which attenuate each alternative electron transfer rate are apparently different. Interestingly, while the rates of each of these alternative electron transfer reactions increase with trimethylamine concentration to a limiting value, we observed that the faster phase dominated at low substrate concentra-

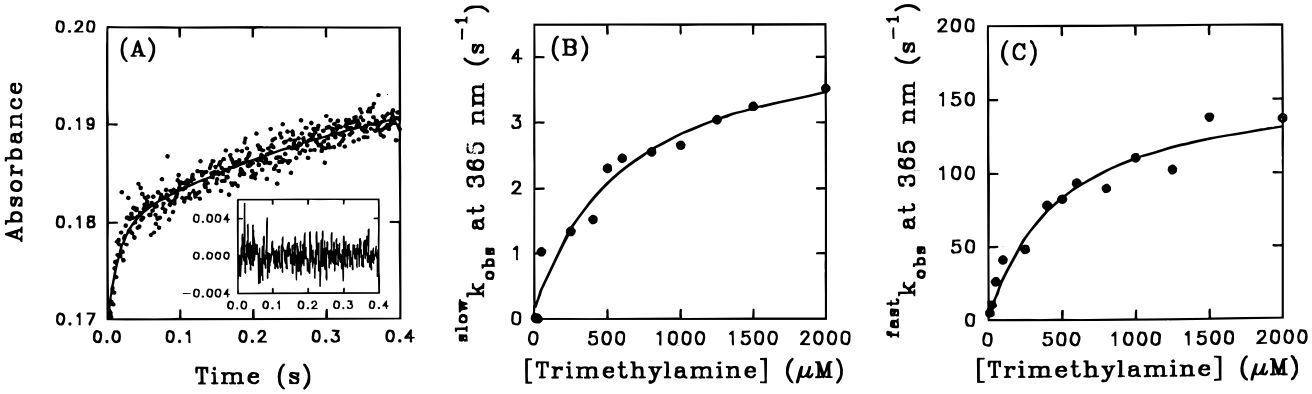


FIGURE 3: (A) Conversion of reduced $FMNH_2$ to FMN semiquinone in TMADH as monitored by the increase in absorbance at 365 nm. TMADH (3 μM) was mixed with trimethylamine (800 μM) in 100 mM potassium phosphate buffer (pH 7.5) at 30 °C. The solid line represents the fit of these data to eq 2, which for this data set gave values for $^{fast}k_{obs}$ of 75 ± 10 s^{-1} and for $^{slow}k_{obs}$ of 2.1 ± 0.01 s^{-1}. The inset shows a plot of the residuals for this fit. (B) Plot of $^{slow}k_{obs}$ against substrate concentration. The values of $^{slow}k_{obs}$ were determined at several concentrations of substrate. The solid line represents the fit of these data to eq 4. (C) Plot of $^{fast}k_{obs}$ against substrate concentration. The values of $^{fast}k_{obs}$ were determined at several concentrations of substrate. The solid line represents the fit of these data to eq 4.

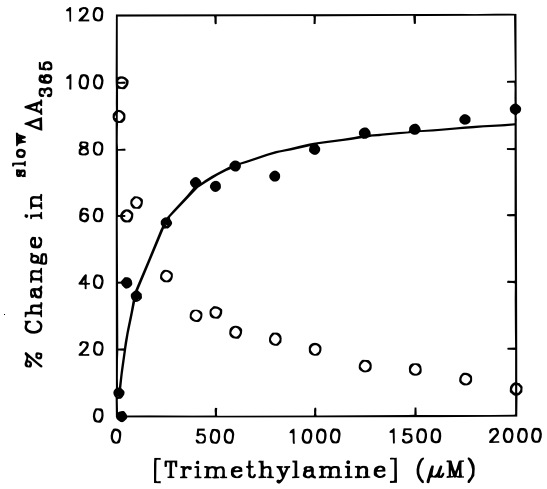


FIGURE 4: Dependence on substrate concentration of the proportion of the absorbance change at 365 nm attributable to $^{fast}k_{obs}$ and $^{slow}k_{obs}$. The percentage of the total amplitude of the absorbance change at 365 nm attributable to the slower (●) and faster (○) rate constant was determined for the data described in Figure 3 and plotted against trimethylamine concentration. The solid line represents the fit of the data for the slower rate to eq 9.

tions, while the slower phase dominated at high concentrations. The amplitude of the absorbance change at 365 nm that corresponds to the faster rate decreases with increasing substrate concentration, and the amplitude of the absorbance change that corresponds to the slower rate increases with increasing substrate concentration (Figure 4). These data represent a titration of the extent to which each of the two alternative intramolecular electron transfer reactions proceeds and were analyzed as though they described a binding event. The amplitude of the absorbance change observed for the slower rate constant was fit to eq 9, which describes ligand

$$^{slow}\Delta A_{365} = \frac{C[S]}{K_d + [S]} \quad (9)$$

binding to a single site, where [S] is the concentration of the trimethylamine and C is the binding capacity in terms of maximum absorbance change, $^{slow}\Delta A_{365}$ (Figure 4). Analysis of these data yielded a K_d for the binding of trimethylamine to this regulatory second site of 148 ± 32 μM . This K_d is statistically different than the K_d for substrate binding to the active site which was determined from the analysis of the data shown in Figure 2 to be 36 ± 28 μM. The apparent K_d obtained from the analysis in Figure 4 does not correspond to trimethylamine binding to the catalytic site of oxidized TMADH. It may correspond to binding to a distinct allosteric site or possibly to the active site of the reduced enzyme. The binding of the second trimethylamine which is not converted to product appears to cause a switching of alternative mechanisms of regulation of the rate of the intramolecular electron transfer reaction from $FMNH_2$ to $[4Fe\text{-}4S]^{2+}$. The likely explanation for the biphasic kinetics of the intramolecular electron transfer is that two different TMADH species are present, one with and one without trimethylamine bound to the second noncatalytic site.

Steady-State Kinetic Assays of the Reductive Half-Reaction. The determination of the steady-state kinetic parameters of TMADH is complicated by an unusual type of substrate inhibition that has been observed with both nonphysiological electron acceptors and ETF. The concentration dependence of the initial rate of reaction of TMADH was determined using PES as an electron acceptor. These data did not fit to either the standard Michaelis−Menten equation (eq 10,

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (10)$$

$$v = \frac{V_{max}[S]}{K_m + [S]\left(1 + \frac{[S]}{K_i}\right)} \quad (11)$$

Figure 5A) or the eq which describes typical uncompetitive substrate inhibition (eq 11, Figure 5A). After deliberate analysis, a kinetic model (Scheme 1) which describes two different binding sites, one active and one allosteric, for the substrate per enzyme subunit was found to be the simplest model that described the steady-state kinetic data (Figure 5B). The initial velocity eq for the model in Scheme 1 is given in eq 12. More complicated models could be derived which also described the data, including the rebinding of a second substrate molecule to the active site of the reduced

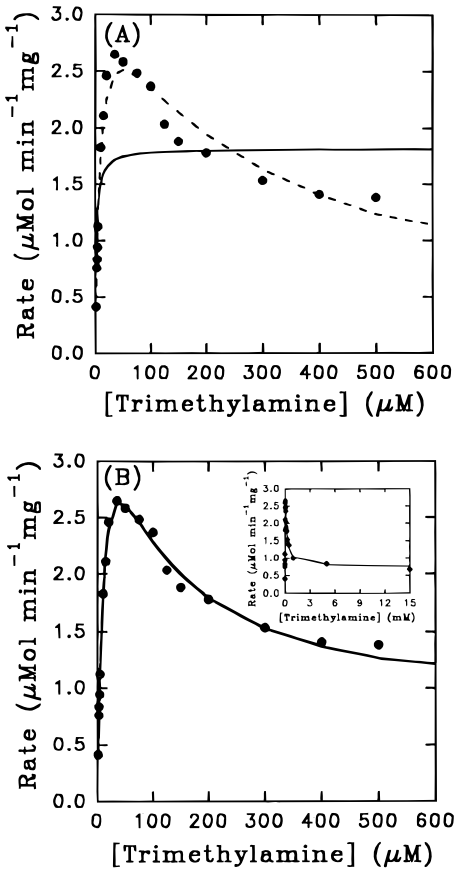
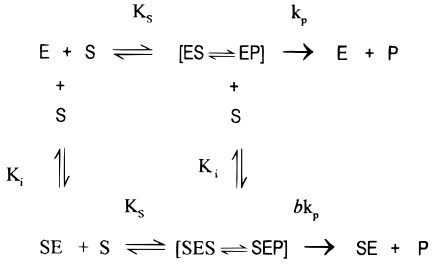


FIGURE 5: Steady-state kinetic analysis of the reaction of TMADH with trimethylamine. (A) The lines represent the poor fits of these data to eqs 10 (—) and 11 (- - -). (B) The solid line represents the fit of the data to eq 12. The inset shows the same fit at much higher concentrations of trimethylamine.

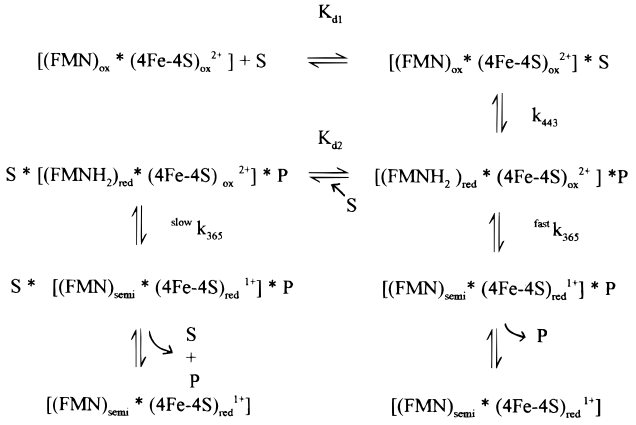
Scheme 1



enzyme. Analysis using the rate eqs derived for these models, however, did not improve the fit of the data obtained with eq 12. These more complex models contained additional unknown variables. Although good fits of the data could be obtained, the values of some of the unknown parameters were dependent upon the initial estimates used to fit the data, and the fitted values also exhibited very large standard errors. The model which is described in Scheme 1 was the simplest for which it was possible to obtain a unique set of fitted values for the unknown parameters.

$$v = \frac{V_{max}\left(\frac{1}{b} + \frac{[S]}{K_i}\right)}{1 + \frac{K_s}{[S]} + \frac{K_s}{K_i} + \frac{[S]}{K_i}} \qquad (12)$$

Scheme 2



For the model described in Scheme 1, the theoretical V_{max} ($k_p[E]_T$), which would only be possible in the absence of substrate inhibition, is never observed. The limiting velocity that is observed at the highest substrate concentrations is equal to $bk_p[E]_T$, where b represents the factor by which the intrinsic maximum velocity is affected. A distinction between this model and simple substrate inhibition models is that in the latter the SES intermediate is a dead-end inactive complex and the rate will eventually go to zero as substrate concentration increases. For TMADH, binding of a second substrate molecule results in an enzyme form with reduced but finite activity. Product is formed from both the ES or SES complexes, but at different rates. Analysis of the data using eq 12 yielded an observed V_{max} ($bk_p[E]_T$) of 0.8 ± 0.05 μmol min^{-1} (mg of enzyme)$^{-1}$ (k_{cat} = 1.1 s^{-1}), a K_s of 20 ± 5 μM, a K_i of 52 ± 15 μM, and a value of b of 0.1. The theoretical V_{max} ($k_p[E]_t$) is 8.0 μmol min^{-1} (mg of enzyme)$^{-1}$ (k_{cat} = 11 s^{-1}). These results suggest that there are two separate trimethylamine binding sites per subunit of TMADH, the primary active site and a second lower affinity inhibitory site. Thus, evidence for two distinct substrate binding sites on TMADH may be inferred from the results of steady-state as well as transient kinetic studies.

DISCUSSION

A kinetic mechanism proposed for the reductive half-reaction of TMADH is depicted in Scheme 2. This model describes the binding of a second molecule of trimethylamine to TMADH which causes a switching of mechanisms which regulate the observed rates associated with the intramolecular electron transfer. The observed rate constant at 443 nm describes the reduction of FMN. The slower and faster observed rate constants recorded at 365 nm represent electron transfer from the reduced flavin to the oxidized iron−sulfur center in the presence and absence of a second molecule of trimethylamine bound to a noncatalytic site. Each rate strictly describes the formation of the flavinsemiquinone. The development of the spin-coupled triplet state is not addressed in this model (discussed below). The point in the reaction when P is released is not known and may be different than is shown in Scheme 2. Product is likely bound in the absence of the second S because $^{fast}k_{365}$ is slower than the intrinsic electron transfer rate, as determined from pH-jump experiments (Rohlfs & Hille, 1991) in the absence of substrate or product. It is possible, however, that the substrate-reduced

TMADH without bound product is not equivalent to the dithionite-reduced TMADH used in those studies. Scheme 2 indicates that the second S and P are bound at the same time. As discussed earlier, we acknowledge that our results do not exclude the possibility that the $^{slow}k_{365}$ might instead be due to binding of a second molecule of substrate at the active site of reduced TMADH which displaces bound product. For the latter alternative to be possible, however, the rates of product release and rebinding of the second substrate molecule would have to be faster than the rate of intramolecular electron transfer from the reduced flavin to the iron−sulfur center.

It must be noted that an alternative model was recently proposed (Rohlfs & Hille, 1994) for the reductive half-reaction of TMADH on the basis of data obtained from transient kinetic studies with diethylmethylamine, a poor alternative substrate. That model assumes that the slowest observed rate for the intramolecular electron transfer ($^{slow}k_{obs}$ at 365 nm) is due to binding of a second molecule of diethylmethylamine to the active site. It differs from Scheme 2, primarily, in that it proposes that $^{slow}k_{365}$ describes the development of the triplet state from the noninteracting substrate-reduced radical form of TMADH. Our data with trimethylamine, as well as certain previously published data, are more consistent with the model presented in Scheme 2. Titration of TMADH with trimethylamine [Figure 5 in Steenkamp et al. (1978a)], and with diethylmethylamine [Figure 4B in Rohlfs and Hille (1994)], was simultaneously followed by absorbance and EPR spectroscopy and showed that the absorption spectra of nontriplet flavinsemiquinone and that of the triplet state (measured by the development of the half-field signal at $g = 4$) are essentially the same. From those data, it can be seen that one cannot distinguish, by monitoring absorbance changes at 365 nm, between the triplet state and the noninteracting flavinsemiquinone. Furthermore, freeze−quench EPR studies using trimethylamine [Figure 3C in Steenkamp and Beinert (1982b)] and diethylmethylamine [Figure 5 in Rohlfs and Hille (1994)] show that the formation of the $g = 4$ EPR signal after mixing of the substrate exhibits a biphasic time course. This seems to be consistent with our biphasic kinetic data which we interpret as representing alternative routes for flavinsemiquinone formation. Significantly, we are able to attribute full development of the spectral change at 365 nm to either of the two alternative rates at the low and high ends of the titration curve shown in Figure 4. This strongly suggests two alternative processes each capable of proceeding to completion. It is possible that different mechanisms may be operative with diethylmethylamine and trimethylamine as substrates. We feel, however, that our data with trimethylamine are more consistent with the model described in Scheme 2.

Correlation between steady-state and transient kinetic models and data is supported by the kinetically determined binding constants. The K_s value determined from the steady-state analysis is statistically equal to the K_d value for trimethylamine binding to the active site which was obtained from the concentration dependence of k_{obs} at 443 nm. The K_d value for trimethylamine binding to the noncatalytic site, which was determined from the analysis of the amplitudes of the absorbance changes corresponding to $^{fast}k_{365}$ and $^{slow}k_{365}$, was 148 ± 32 μM, compared to the K_i value for trimethylamine binding of 52 ± 15 μM. These values are also similar, but not identical. The difference may be due to the fact that the steady-state model in Scheme 1 is an oversimplification which neglects steps which contribute to K_i but which are not distinguishable in the steady-state assay. The observation that TMADH exhibits substrate inhibition in the steady-state reaction raises the possibility that an allosteric binding site for trimethylamine, which is responsible for substrate inhibition, is the same as an allosteric site which regulates the rate of intramolecular electron transfer from $FMNH_2$ to $[4Fe\text{-}4S]^{2+}$. The theoretical steady-state value of k_{cat} (11 s^{-1}) is significantly less than the limiting value for $^{fast}k_{365}$ (160 s^{-1}). However, the limiting value for $^{slow}k_{365}$ (4 s^{-1}) is less than the theoretical k_{cat} , and is close to the value for the observed k_{cat} (1.1 s^{-1}) which is attenuated by substrate inhibition. These values are not identical, but their closeness suggests that in the presence of high concentrations of substrate $^{slow}k_{365}$ is at least partially rate-limiting in the steady state and may account for the observed substrate inhibition.

For redox reactions involving proteins, the actual meaning of the kinetically determined electron transfer rate constant (k_{ET}) must be interpreted with caution. One must acknowledge the possibility that protein dynamics (i.e. transient formation of unstable conformational intermediates) or catalytic events (i.e. protonation/deprotonation) may be contributing to the observed k_{ET} (Bishop & Davidson, 1995). In the kinetic models that are used to analyze the transient kinetic data, any spectroscopically invisible events subsequent to binding and preceding the spectral change associated with the change in redox state will be reflected in k_{ET}. For example, these events could include substrate-induced or redox-linked protein conformational changes. If any of these events is slower than the actual electron transfer rate, then the reaction will be gated (Hoffman & Ratner, 1987) and k_{ET} will be equal to the rate of that process, not the actual electron transfer step. Even when the actual electron transfer step is rate-determining, it will not be equal to the true k_{ET} if such a preceding step is rapid but very unfavorable (Brunschwig & Sutin, 1989; Harris et al., 1994). Given the relatively close proximities of the two redox centers in TMADH, one would intuitively expect the intramolecular k_{ET} to be much greater than either $^{fast}k_{365}$ or $^{slow}k_{365}$. Rates of intramolecular electron transfer within TMADH, in the absence of trimethylamine, have been measured using a pH-jump technique (Rohlfs & Hille, 1991). The values of k_{ET} obtained in that study were significantly greater than those reported in this paper. Thus, it is very likely that the limiting values of $^{fast}k_{365}$ and $^{slow}k_{365}$ reported here represent the rates of electron transfer reactions which are either gated or coupled to some non-electron transfer event. What is remarkable about TMADH is that two different apparent values of k_{ET} are observed depending upon (i) whether substrate or product is present at the active site of oxidized TMADH and (ii) whether a second molecule of substrate is also bound to TMADH. Neither of these rates appears to be that of a true electron transfer reaction. Instead, two different mechanisms for gating or attenuating the electron transfer rate are operative in this enzyme, depending upon whether one or both of the substrate binding sites is occupied. The binding of substrate to an allosteric site may act as a switch between alternative mechanisms for regulation of the rate of electron transfer. The basis for this phenomenon will be the subject of future studies.

Certain properties of the intramolecular electron transfer reaction within TMADH which are characterized here are analogous to interesting observations recently reported for other redox enzymes. Electron transfer from FMN to a [2Fe-2S] center in phthalate dioxygenase reductase occurs at a rate of only 35 s^{-1} despite the fact that the two redox centers are separated by only about 5 Å (Gassner et al., 1994). Consequently, the relatively slow intramolecular electron transfer between flavin and iron−sulfur centers which is observed with TMADH is not an isolated phenomenon. Examples of intramolecular electron transfer reactions whose rates are controlled by ligand binding have also been reported. The presence of excess pyruvate, a reaction product, significantly reduces the rate of intramolecular electron transfer between FMN and heme in flavocytochrome b_2 (Hazzard et al., 1994). In ascorbate oxidase, oxygen enhances the rate of intramolecular electron transfer between type I and type 3 coppers (Farver et al., 1994). Therefore, allosteric control of k_{ET} by trimethylamine in TMADH may be representative of a more widespread motif for regulating biologic electron transfer reactions.

A final point which is raised by the observation of substrate inhibition in TMADH and attenuation of k_{ET} by excess substrate is whether this may have any biological relevance. During metabolism, the methylotrophic bacterium *M. methylotrophus* W3A1 converts the carbon source, trimethylamine, to dimethylamine and formaldehyde. Dimethylamine is subsequently converted to methylamine and formaldehyde, and methylamine is then oxidized to ammonia and formaldehyde. The formaldehyde is assimilated by either the ribose phosphate or serine pathway (Colby & Zatman, 1975). Formaldehyde, however, is also potentially very toxic to the cell. When the bacterium is presented with high concentrations of trimethylamine, it is possible that the production of formaldehyde by TMADH might exceed the capacity of the cell to assimilate formaldehyde. This would potentially be fatal. Very often, key metabolic enzymes are regulated by the concentration of the product of an enzyme reaction by a negative feedback mechanism. With this type of control, when the level of the regulating metabolite reaches a sufficiently high concentration, it inhibits the key enzyme by binding to it at some regulatory site which is distinct from the catalytic site. In the case of trimethylamine metabolism, it may not be wise to use formaldehyde as a regulatory signal because of its potential toxicity. Alternatively, TMADH may have evolved a mechanism to avoid accumulation of this toxic compound by adopting this mechanism of substrate inhibition to depress the levels of formaldehyde production when excess substrate is present.

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