The Reaction of Trimethylamine Dehydrogenase with Trimethylamine

(Received for publication, July 21, 1998, and in revised form, December 3, 1998)

Mei-Huei Jang‡, Jasvir Basran§, Nigel S. Scrutton§, and Russ Hille¶§

From the ‡Department of Medical Biochemistry, The Ohio State University, Columbus, Ohio 43210 and the §Department of Biochemistry, University of Leicester, University Road, Leicester LE1 7RH, United Kingdom

The reductive half-reaction of trimethylamine dehydrogenase with its physiological substrate trimethylamine has been examined by stopped-flow spectroscopy over the pH range 6.0–11.0, with attention focusing on the fastest of the three kinetic phases of the reaction, the flavin reduction/substrate oxidation process. As in previous work with the slow substrate diethylmethylamine, the reaction is found to consist of three well resolved kinetic phases. The observed rate constant for the fast phase exhibits hyperbolic dependence on the substrate concentration with an extrapolated limiting rate constant \( k_{\text{lim}} \) greater than 1000 s\(^{-1}\) at pH above 8.5, 10 °C. The kinetic parameter \( k_{\text{lim}}/K_s \) for the fast phase exhibits a bell-shaped pH dependence, with two \( K_a \) values of 9.3 ± 0.1 and 10.0 ± 0.1 attributed to a basic residue in the enzyme active site and the ionization of the free substrate, respectively. The sigmoidal pH profile for \( k_{\text{lim}} \) gives a single \( K_a \) value of 7.1 ± 0.2. The observed rate constants for both the intermediate and slow phases are found to decrease as the substrate concentration is increased. The steady-state kinetic behavior of trimethylamine dehydrogenase with trimethylamine has also been examined, and is found to be adequately described without invoking a second, inhibitory substrate-binding site. The present results demonstrate that: (a) substrate must be protonated in order to bind to the enzyme; (b) an ionization group on the enzyme is involved in substrate binding; (c) an active site general base is involved, but not strictly required, in the oxidation of substrate; (d) the fast phase of the reaction with native enzyme is considerably faster than observed with enzyme isolated from *Methylophilus methylotrophus* W3A4, that catalyzes the oxidative demethylation of trimethylamine to dimethylamine and formaldehyde (presumably through an imine intermediate that spontaneously hydrolyzes once dissociated from the enzyme),

\[
(\text{CH}_3)_3\text{N} + \text{H}_2\text{O} \rightarrow (\text{CH}_3)_3\text{NH} + \text{CH}_3\text{O} + 2\text{H}^+ + 2e^- \tag{1}
\]

The enzyme is a homodimeric protein having a subunit molecular mass of 83 kDa, with each subunit containing a covalently linked 6-S-cysteinyl FMN cofactor and a bacterial ferredoxin type 4Fe/4S center; each subunit also possesses 1 equivalent tightly bound ADP of unknown function (1–7). The physiological electron acceptor for TMADH is an electron transferring flavoprotein (ETF), an \( \alpha \beta \) dimer of molecular mass 62 kDa. ETF contains 1 equivalent of FAD, which cycles between oxidized and (anionic) semiquinone oxidation states (8), and 1 equivalent AMP, whose function remains unclear (9).

Full reduction of TMADH requires three electrons per subunit, two for reduction of the FMN and a third for reduction of the 4Fe/4S center, but only two reducing equivalents are removed from substrate during catalysis. The distribution of reducing equivalents within two-electron reduced enzyme generated by reduction with excess substrate favors the formation of flavin semiquinone and reduced 4Fe/4S center, with the magnetic moments of the two paramagnetic centers interacting strongly to give a spin-interacting state (a triplet state), which is distinguished by a complex EPR signal centered near \( g \sim 2 \) and an unusually intense half-field \( g \sim 4 \) signal (10–16). Reduction of enzyme with dithionite in the presence of the substrate analog and inhibitor tetramethylammonium chloride (TMAC), or by titration with dithionite at high pH also generates this characteristic spin-interacting state.

Previous stopped-flow and freeze-quench EPR kinetic studies have demonstrated that the reaction of TMADH with trimethylamine consists of three kinetic phases. The first phase involves a very rapid bleaching of the enzyme-bound FMN and has been only poorly characterized owing to its rapid rate \( t_{1/2} \lesssim 2 \) ms at 500 \( \mu \)M trimethylamine in 0.1 M pyrophosphate buffer, pH 7.7, 18 °C; Ref. 10). This fast phase is followed by two slower kinetic phases with spectral changes reflecting intramolecular electron transfer from reduced flavin to the 4Fe/4S center to give flavin semiquinone and reduced 4Fe/4S center (10–14, 17). More recently, the reductive half-reaction has also been investigated using a slow substrate, diethylmethylamine, and the reaction also exhibits three kinetic phases (18). On the basis of the kinetic studies with diethylmethylamine, an overall reductive half-reaction mechanism for TMADH has been proposed (15, 18): the fast phase represents the two electron reduction of the flavin cofactor (oxidation of the substrate) with simultaneous formation of a covalent substrate-flavin intermediate; the intermediate phase reflects intramolecular electron transfer from reduced flavin to the 4Fe/4S center, generating flavin semiquinone and reduced 4Fe/4S center with intrinsic rapid

Trimethylamine dehydrogenase (TMADH, EC 1.5.99.7) is an iron-sulfur containing flavoprotein isolated from the bacterium *Methylophilus methylotrophus* W3A4 that catalyzes the oxidative demethylation of trimethylamine to dimethylamine and formaldehyde (presumably through an imine intermediate that spontaneously hydrolyzes once dissociated from the enzyme),

\[
(\text{CH}_3)_3\text{N} + \text{H}_2\text{O} \rightarrow (\text{CH}_3)_3\text{NH} + \text{CH}_3\text{O} + 2\text{H}^+ + 2e^- \tag{1}
\]
The Reaction of Trimethylamine Dehydrogenase with Trimethylamine

electron transfer rate limited by the decay of the covalent adduct (15); and the slow phase involves dissociation of product and binding of a second substrate molecule, which perturbs the electron distribution in the partially reduced enzyme and facilitates formation of the spin-interacting state. Most recently, the reaction with trimethylamine has been re-examined with a considerably slower rate constant for the first phase of the reaction being reported despite the fact that the experiment was performed at 30 °C (19). In addition, a kinetic mechanism involving an additional inhibitory substrate-binding site was proposed, although there is no independent evidence for the existence of such an additional substrate binding site.

In an effort to clarify the discrepancies concerning the kinetic behavior of TMADH and to further elucidate its reaction mechanism, a comprehensive pH dependence study of the enzyme reaction with trimethylamine has been performed, with particular attention paid to the fast phase of the reaction. By working at 10 °C, we have been able to characterize the substrate concentration and pH dependence of the fast phase of the reaction. We find that this phase is indeed very rapid, with an extrapolated limiting rate constant greater than 1000 s⁻¹ at pH 8.5 and higher, even at 10 °C. Both rapid kinetic and steady-state results can be readily explained by a proposed kinetic mechanism involving two alternative catalytic cycles depending on the relative availability of substrate and electron acceptor. We conclude that the kinetic behavior of the enzyme can be adequately described from a consideration of the known properties of the enzyme without invoking a second substrate-binding site.

EXPERIMENTAL PROCEDURES

Enzyme Purification—M. methylotrophus W1A3 was grown on trimethylamine as sole carbon source and TMADH was purified essentially as described by Steenkamp and Mallinson (1) with the exception that Sephadex S-200 rather than Sephadex G-200 was used for the gel filtration step in the purification. Enzyme concentrations were determined from the 442 nm absorbance of oxidized enzyme using an extinction coefficient of 27.3 mM⁻¹ cm⁻¹ (6). The enzyme was found to be stable over the pH range 6–11 used in the present study.

Trimethylamine—Hypersoluble—trimethylamine hydrochloride solutions were obtained from Sigma. TMAC was from Aldrich and dried prior to use employing an indirect heated drying tube containing phosphorous pentoxide in a side chamber. Phosphate and pyrophosphate buffers were obtained from Sigma and boric acid from Jenneke Chemical Co. (inorganic buffers are necessary since most organic buffers contain substituted amines, and either inhibit the enzyme or serve as substrates). Sodium dithionite and dithionite from Virginia Chemicals. Phenazine ethosulfate and 2,6-dichlorophenolindophenol were from Sigma, and benzyl viologen (0.5 M) was from FMC.

Pre-steady-state Experiments—Kinetic experiments were carried out using a Kinetic Instrument Inc. stopped-flow apparatus equipped with a Kinetic Instrument Inc. data collection system. Anaerobic solutions of oxidized TMADH were prepared in the following way. A concentrated sample of oxidized enzyme was passed through a Sephadex G-25 column equilibrated with 0.1 M solution of an appropriate buffer adjusted to the desired pH (phosphate buffer for pH 6.0–7.5, pyrophosphate buffer for pH 8.0–8.5, borate buffer for pH 9.0–11.0). The enzyme solution was then diluted with buffer to give a final concentration of 10–20 μM, placed into a tonometer equipped with a side arm cuvette, and made anaerobic by repeated evacuation and flushing with O₂-free argon. Trimethylamine hydrochloride solutions prepared in the same buffer were placed in 20-ml glass syringes and made anaerobic by bubbling with O₂-free argon for at least 15 min. Kinetic transients were monitored as transmission voltages collected by a high speed A/D converter and converted to absorbance changes by the double integration method using enzyme and substrate concentrations of 18 and 500 μM, respectively.

RESULTS

Reductive Half-reaction Kinetics of TMADH with Trimethylamine—The reductive half-reaction of TMADH with trimethylamine has been studied by stopped-flow spectroscopy at 10 °C over the pH range 6.0–11.0. The reaction is found to consist of...
three kinetic phases: a fast process followed by two slower ones (hereafter designated fast, intermediate, and slow, respectively). Kinetic transients observed at 450 nm consist principally of the fast kinetic phase throughout the pH range examined here, although a small absorbance change associated with the two slower phases can also be seen (Fig. 1A). The fast phase of the reaction at 10 °C is sufficiently slow as to permit accurate determination of the rate constants for this phase. At 365 nm, the kinetic transients consist essentially of the two slower phases with only a very small spectral change associated with the fast phase (Fig. 1B). While kinetic transients obtained at 520 nm contain substantial contributions from all three kinetic phases (Fig. 1C).

Under the pseudo-first order conditions used in the present experiments, the rate constant of the fast phase exhibits a hyperbolic substrate concentration dependence at all pH values examined (data not shown), consistent with the formation of a Michaelis complex between enzyme and substrate prior to bleaching of the flavin (20). The data can be fitted to the hyperbolic equation: 

\[ k_{\text{obs}} = \frac{k_{\text{lim}} [S]}{K_d + [S]} \]

The pH dependence of \( k_{\text{lim}} \) and \( k_{\text{lim}}/K_d \) are shown in Fig. 2, A and B. It is seen that this phase is quite rapid, with extrapolated \( k_{\text{lim}} \) values greater than 1000 s\(^{-1}\) above pH 8.5 even at 10 °C. The pH dependence of \( k_{\text{lim}} \), reflecting the progress of the enzyme-substrate complex through the first irre-

\[ 2 \text{ The dead-time of the stopped-flow apparatus used in the present study is approximately 1.0 ms and it is estimated that about 40–60\% of the spectral change associated with the fast kinetic phase of the reaction occurs in the dead-time of the rapid mixing apparatus (depending on the pH and/or substrate concentrations used). Fortunately, with the large spectral changes associated with the fast phase (\( \Delta \epsilon = 12 \text{ mm}^{-1} \text{ cm}^{-1} \)), the high enzyme concentrations used in the present study (10–20 \( \mu \text{M} \)), and the 2-cm light path of the mixing cell, the observed absorbance change from which \( k_{\text{obs}} \) has been obtained is in the range 0.06 to 0.3 absorbance units. The reliability of the obtained rate constants are further improved by performing more than three independent measurements under each experimental condition and averaging the results.}
versatile step of the reaction (20), exhibits simple sigmoidal behavior with a single pK_a of 7.1 ± 0.2. By contrast, the pH profile of k_{lim}/K_d is bell-shaped and a fit using the general equation for such a profile ("Experimental Procedures," Equation 4) yields pK_a values of 9.3 ± 0.1 and 10.0 ± 0.1. Since k_{lim}/K_d tracks the reaction of free substrate and free enzyme (20) and the latter pK_a value agrees well with that for free trimethylamine (9.81; Ref. 23), we thus attribute the latter to the ionization of free substrate and the former to an ionizable group in the enzyme active site. The present results are similar to those obtained previously with diethylmethylamine as substrate, although, significantly, the pK_a associated with free substrate was not identified in this earlier work as the data were not extended to sufficiently high pH (18). To confirm the bell-shaped behavior in the k_{lim}/K_d plot, we have reexamined the reaction of TMADH with diethylmethylamine to include data for pH 9.5 and 11.0. The results are shown in Fig. 2, C and D, and the ionization of substrate is clearly evident in the pH profile for k_{lim}/K_d as the descending limb of the bell-shaped curve at high pH.

Several difficulties are associated with analysis of the intermediate phase of the reaction. Below pH 7.0, the spectral change associated with this phase is small at most wavelengths, making it difficult to resolve the two phases (given the large spectral change associated with the fast phase, there is little compromise in the determination of its rate constant). Also, above pH 9.5 the rate constants for the intermediate and slow phases approach each other at high substrate concentrations and prevent reliable determination of rate constants for either phase. As a result, only data over the range pH 7.5–9.5 can be analyzed (the result for pH 8.0 is shown in Fig. 3A). At all pH values examined, the observed rate constant for intermediate phase is substrate concentration dependent, but exhibits a pronounced substrate inhibition pattern. The maximum apparent rate constant is essentially independent of pH (about 20 s^{-1}). At a given pH, the rate constant decreases as substrate concentration increases (except for pH 7.5 where the rate constant slightly increases as the substrate concentration increases at [TMA] ≤ 500 μM). The degree of apparent substrate inhibition increases with pH, with the concentration required to give a 50% reduction in rate constant decreasing from ~2 mM at pH 7.5 to ~0.3 mM at pH 9.5.

As with the intermediate phase, it is difficult to obtain reliable rate constants for the slow phase of the reaction. Generally, the magnitude of the spectral change associated with the slow phase decreases as pH increases, and as a result there is poor resolution from the intermediate phase at high pH. Therefore, we were only able to determine the substrate concentration dependence of the slow phase between pH 6.0 and 8.0. The results of the experiment at pH 8.0 are shown in Fig. 3B, where it is evident that the observed rate constant again exhibits substrate inhibition. At each pH, the rate constant increases at low substrate concentrations, then decreases at higher concentrations. The maximum rate constant increases with pH (from ~0.03 s^{-1} at pH 6.0 to ~2 s^{-1} at pH 8.0) while the substrate concentration that gives the maximum rate constant decreases

---

3 In the accompanying study (33) of the Y169F mutant of TMADH, the k_{lim} pH profile was fitted to two pK_a values rather than a single pK_a, as here. Given the magnitude of the standard error in the present data, we are not able to unambiguously ascertain whether a second pK_a exists for this plot. Also, we do not eliminate the possibility here that the pK_a evident in the pH profile for k_{lim} may arise from enzyme-bound substrate. This would require that enzyme bind the protonated form of substrate more tightly than the neutral form, but that the Michaelis constant for the latter break down more rapidly.

4 There is a potential concern that a portion of the observed kinetic effect might be due to ionic strength effects on the reaction at high substrate concentration. The reductive half-reaction of TMADH with trimethylamine has also been studied at pH 7.5, 10 °C in the presence of 0.2 N potassium chloride and same kinetic behavior is observed as reported here.
Inhibitory effect of excess substrate is more dramatic at higher pH, as seen for the intermediate phase.

Steady-State Kinetics of TMADH with Trimethylamine—To correlate with the rapid kinetic work, the steady-state kinetic behavior of TMADH with trimethylamine has been studied at pH 8.0, 10 °C. The substrate concentration dependence of initial velocity over a TMA concentration range of 5 μM to 5 mM is shown in Fig. 3C. As seen previously (14), excess substrate inhibition is observed with the initial velocity increasing at low substrate concentration (up to approximately 50 μM) and then decreasing as substrate concentration further increases. The inhibition at which substrate inhibition is observed, however, is much lower than is the case for the reductive half-reaction experiments described above.

Spectral Changes Associated with the Reaction of TMADH with Trimethylamine—The spectral changes associated with each kinetic phase seen in the reductive half-reaction have been determined by stopped-flow spectroscopy with the results shown in Fig. 4. Under the present experimental conditions ([TMADH] = 18 μM, [TMA] = 500 μM, 0.1 M pyrophosphate buffer, pH 8.0, 10 °C), the three kinetic phases are well resolved (k_{fast} = 500 s^{-1}; k_{int} = 17 s^{-1}; k_{slow} = 1.6 s^{-1}) and the spectral changes associated with each phase readily determined. That for the fast phase agrees well with that reported previously by Beinert and co-workers obtained at pH 7.7 (10) and is consistent with either true reduction of the flavin cofactor or formation of a substrate-flavin covalent intermediate, as proposed previously (18). As seen previously with diethylmethyldamine (18), the spectral changes associated with the intermediate and slow phases are essentially the same, only the relative amplitudes of the two phases change as a function of pH (18). The total absorbance change associated with the two phases seen here is also comparable to that reported by Beinert and co-workers (Ref. 10; working at pH 7.7).

The reaction rates and absorbance changes associated with each phase are shown in Figs. 3A-C and 4A-B. The reaction rates were determined by fitting kinetic transients measured at 365 nm and represent the mean of at least three independent measurements. The absorbance changes were determined by stopped-flow spectroscopy and represent the mean of at least three independent measurements performed at a given substrate concentration. The error bars for each data point represent the standard deviation of the mean.

**FIG. 3.** Substrate concentration dependence of k_{fast}, k_{int}, and k_{cat} at pH 8.0. A, trimethylamine concentration dependence of the rate constants for the fast phase of the reductive half-reaction, k_{fast}. B, trimethylamine concentration dependence of the rate constants for the slow phase of the reductive half-reaction, k_{slow}. C, substrate concentration dependence of the steady-state kinetic parameter k_{cat}. The symbols in A and B represent values of rate constants obtained at a specific substrate concentration and represent the mean of at least three independent measurements. The symbols in C represent values of k_{cat} obtained at a specific substrate concentration and represent the mean of at least three independent measurements performed at a given substrate concentration. The error bars for each data point represent the standard deviation of the mean.

**FIG. 4.** Spectral intermediates observed during the reduction of TMADH by trimethylamine. Reaction conditions are the same as described in the legends to Fig. 1. A, the kinetic difference spectra obtained from the absorbance change associated with the absorbance change associated with the fast kinetic phase (reaction intermediate formed by the fast kinetic phase) minus (oxidized enzyme), the absorbance change associated with the intermediate kinetic phase (reaction intermediate formed by the intermediate kinetic phase) minus (reaction intermediate formed by the fast kinetic phase), and the absorbance change associated with the slow kinetic phase (final spectrum) minus (spectral change observed in the conclusion of the intermediate kinetic phase). B, the visible spectra of oxidized TMADH (solid line), the enzyme species present at the conclusion of the fast kinetic phase (square), the enzyme species present at the end of the intermediate kinetic phase (circles), and the final form of the enzyme observed at the completion of the reductive half-reaction (diamond).
DISCUSSION

We have examined the reductive half-reaction of TMADH with trimethylamine as a function of pH. The pH profile for $k_{\text{lim}}/K_d$ from the fast phase of the reaction is bell-shaped and fits to the data yield $pK_a$ values of 9.3 ± 0.1 and 10.0 ± 0.1, which we attribute to a basic residue on the free enzyme and the ionization of the free substrate, respectively. The implication is that substrate must be protonated to bind to the protein, an observation consistent with the proposal that TMADH binds its substrate through cation-π bonding interactions (25). Examination of the x-ray crystal structure of TMADH indicates the possible candidates for the basic residue are Tyr-169, Tyr-60, and His-172 (24). Tyr-60 may be excluded as a Y169F mutant of TMADH still exhibits the observed ionization (see the Ref. 33), as does an H172Q mutant (although the limiting rate constant at high pH is <10% that seen with wild-type enzyme). This leaves Tyr-60, which with Trp-355 and Trp-264 constitutes the substrate-binding site for the enzyme (25).

Given the only modest pH dependence to $k_{\text{lim}}$ (see below), with the principal basis for the pH dependence of $K_d/ka$ arising from the pH dependence of $K_d$ itself. This being the case, ionization of a group in the substrate-binding site such as Tyr-60 is reasonable to account for the effects observed here. Efforts are presently under way to prepare a Y60F TMADH mutant of TMADH so that this can be tested experimentally.

The pH dependence of $k_{\text{lim}}$ for the fast phase exhibits a $pK_a$ of 7.1 ± 0.2 which may be due to the same enzyme residue as seen in the $k_{\text{lim}}/K_d$ profile (possibly Tyr-60) with a $pK_a$ shift upon substrate binding. This decrease in $pK_a$ upon binding substrate would tend to deprotonate the active site base, thereby putting this group in the proper ionization state to facilitate the reaction, but we cannot at present exclude the possibility that this ionization arises from another active site residue in the enzyme-substrate complex. We emphasize that the low pH asymptote to the $k_{\text{lim}}$ profile is non-zero for both trimethylamine and diethylmethylamine (present study and Ref. 18), indicating that the basic residue observed in the $k_{\text{lim}}$ plot, although important, is not strictly required for catalysis; indeed, it accelerates catalysis only about 6-fold. Even were the protonation step to be fully reversible and this factor of six to underestimate the intrinsic effect (due to a significant equilibrium effect), it is nevertheless evident from the present work that protonation of the responsible group does not seriously compromise breakdown of the E$S$ complex.

The mechanism of amine oxidation as catalyzed by flavoproteins is an issue of considerable controversy at present, particularly as regards the mechanism of action of monoamine oxidase. On the basis of the above discussion, a mechanism based on proton abstraction (Scheme 1a) seems unlikely in the case of TMADH, as protonation of the active site base has only a relative small effect on the observed rate of decay of the enzyme-substrate complex. Based on the behavior of a variety of mechanism-based inhibitors and with the chemical precedent of nonenzymatic mechanisms of amine oxidation, Silverman (26) has advocated a mechanism for monoamine oxidase in which substrate is initially oxidized by single electron transfer to the enzyme flavin to give an amminium cation radical (and anionic flavin semiquinone). The preponderance of the evidence is considered to support a mechanism in which this radical pair first recombines to form a covalent adduct which then decays by β-elimination, as indicated in Scheme 1b. Alternatively, Edmondson (27) has considered a hydrogen atom abstraction mechanism to be preferable for monoamine oxidase (Scheme 1c), based principally on the absence of a significant electronic influence on reaction rate in a homologous series of benzylamine derivative (in conjunction with kinetic isotope work suggesting that the transition state is late rather than early in the course of the reaction). We note here that all proposed mechanisms for monoamine oxidase begin with neutral substrate rather than the protonated form as is shown to be the case here with TMADH. On first principles, we consider it unlikely that the reaction is initiated by a single electron transfer from substrate to the enzyme flavin when substrate is already positively charged, even if the large uphill driving force for the reaction (corresponding to a difference in reduction

---

**Fig. 5.** Half-field EPR spectra of stoichiometric amounts of two-electron reduced TMADH and TMAC. Control sample (upper solid line); 488 μM stoichiometric mixture (__); 300 μM (bold solid line); 200 μM (dark dashes). Inset, percentage of triplet state formation versus mixture concentration. Solid line shows fit of the data to a simple hyperbolic equation with $K_d = 40 \pm 5$ μM. EPR parameters were as described under “Experimental Procedures.”

---

6 J. Basran and N. S. Scrutton, unpublished data.
potentials for donor and acceptor on the order of +1 V) were somehow accounted for. Similarly, a mechanism involving direct nucleophilic attack of the nitrogen lone-pair of substrate on the flavin 4a-carbon (Scheme 1d, also considered in the case of monoamine oxidase; Ref. 28) can be eliminated as a possibility, at least for TMADH, again on the basis of our observation that the reactive form of substrate is protonated. Thus, although the present data do not directly address the mechanism by which C-H bond cleavage occurs, the fact that substrate must be protonated essentially rules out two of the mechanisms shown in Scheme 1, at least in the case of TMADH. Only a mechanism initiated by hydrogen atom abstraction appears to remain fully consistent with the present results. Here, however, the problem is that (as in the case of monoamine oxidase) there is no obvious candidate for the hydrogen atom acceptor. Tyr-60 is part of the substrate-binding site and well situated to act in this capacity, but no tyrosyl (or tryptophanyl) radical has ever been observed with the enzyme, even when treated with strong oxidants such as ferricenium ion. Clearly, much additional work is required to address this issue, and to this end mechanism-based inhibitor studies along the lines of those done previously for monoamine oxidase are presently being pursued with TMADH.

Regardless of the precise mechanism for the first step of the reaction of TMADH with trimethylamine, the present study demonstrates it is indeed very rapid, with extrapolated $k_{\text{lim}}$ values greater than 1000 s$^{-1}$ at pH above 8.5, 10 °C. The magnitude of these rate constants are generally consistent with the half-life for the fast phase at pH 7.7 of 1.5–2 ms reported by Beinert and co-workers (10) when the higher temperature utilized in this earlier study is taken into account. In a more recent study, however, the kinetic properties for the reductive half-reaction of TMADH have been reported that differ significantly from those reported here (19), with limiting rate constants for the fast phase of 230 s$^{-1}$ at pH 7.5, 30 °C (significantly smaller than those reported here). In addition, the present results indicate that the slower phases of the reaction exhibit excess substrate inhibition, while this more recent work reported hyperbolic substrate concentration dependence for both the intermediate and slow phases. The basis for these discrepancies in kinetic behavior appear to have to do with the fact that different substrates are used as carbon source for the cultures from which protein was purified; trimethylamine was used in the present case while dimethylamine was used in the other study. It is known that TMADH isolated from cells grown on dimethylamine exhibits lower specific activity and also has altered spectral properties (29), which have been attributed to chemical modification of the enzyme by impurities in com-
The Reaction of Trimethylamine Dehydrogenase with Trimethylamine

vially available dimethylamine, and/or incomplete flavinylation of the enzyme.9 The present work underscores the importance of working with enzyme isolated from cells grown on the native substrate for the enzyme. In addition, we have recently found that ethylene glycol is a pronounced inhibitor of TMADH,8 and this may also have contributed to the differences in kinetic behavior exhibited by the enzyme in these two studies (ethylene glycol was not used in storage of protein used in the present work).

For the intermediate and slow phases of the reductive half-reaction, we are able to obtain reliable rate constants over only a limited pH range. At pH 7.5, our results are again generally consistent with the previous work of Steenkamp and Beinert (14) done at pH 7.7. A comparison of the rate constants obtained in pre-steady-state studies with $k_{cat}$ from the steady-state analysis indicates that the slow phase is principally rate-limiting in catalysis (although the intermediate phase may also be responsible for the overall catalytic resistance at high substrate concentrations). Excess substrate inhibition is observed in the rapid reaction kinetics for the two slower phases as well as in the steady-state kinetics, but steady-state inhibition is observed at much lower substrate concentrations than either of the two slower phases of the reductive half-reaction (Fig. 3), indicating that other kinetic effects must account for the phenomenon.

By way of understanding the steady-state inhibition of TMADH at high concentrations of substrate, we have noted that TMADH can utilize two alternate catalytic cycles (17): oxidized and two-electron reduced enzyme (an 0/2 cycle) or one- and three-electron reduced enzyme (a 1/3 cycle). This arises from the circumstance that substrate donates two electrons, while ETF takes up only one electron and the enzyme itself can take as many as three electrons. Which cycle predominates in the steady-state depends primarily on the relative concentrations of reducing substrate and electron acceptor. At low TMA and/or low ETF concentrations, the 0/2 cycle is expected to predominate, and conversely at high substrate and/or low ETF concentrations, the 1/3 cycle should be more important. Enzyme turnover in the 1/3 cycle is expected to be slower than that in the 0/2 cycle since substrate binding is known to stabilize the semiquinone form of the flavin in one-electron reduced enzyme (30). Thus, binding of substrate to the partially reduced enzyme (30) such that the flavin center becomes reduced. To the extent that this takes place, oxidation of the bound substrate cannot occur as the flavin is not able to accept a pair of reducing equivalents from substrate. The kinetic effect is equivalent to excess substrate inhibition but, significantly, does not involve a second inhibitory substrate-binding site. An analogous mechanism has also been shown to account for the excess substrate inhibition observed with xanthine oxidase (31). We emphasize that this mechanism for excess substrate inhibition in the steady-state is a necessary consequence of the known properties of TMADH, and is distinct from a model in which a second, inhibitory, substrate-binding site is present in the enzyme, as has been suggested (19). We note that the x-ray crystal structure of TMADH in complex with the substrate analog TMAC gives no indication of the presence of a second substrate-binding site (24, 32). In addition, it has been shown in gel filtration experiments with [14C]trimethylamine that no more than 1 equivalent of substrate is bound to the reduced enzyme (13). The existence of only a single substrate-binding site is further supported by the present EPR studies demonstrating that 1 equivalent of the substrate analog TMAC is sufficient to generate the spin-interacting state in two-electron reduced TMADH. Our results are thus entirely consistent with the x-ray crystallographic data and the earlier gel filtration experiment indicating the presence of a single substrate-binding site on the enzyme.

The results presented here provide several important insights into the mechanism of TMADH and also clarify some discrepancies considering the kinetic behavior of this enzyme in the literature. Studies of related TMADH mutants are presently under way to further elucidate the reaction mechanism of the enzyme and to identify amino acid residues involved in substrate binding and catalysis.

Acknowledgments—We thank Craig Hemann for valuable technical assistance on the EPR studies and Alexander Lazarev for help with the protein purification and pre-steady-state kinetic experiments.

REFERENCES

1. Steenkamp, D. J., and Mallinson, J. (1976) Biochim. Biophys. Acta 429, 705–719
2. Steenkamp, D. J., and Singer, T. P. (1976) Biochim. Biophys. Acta 429, 1269–1295
3. Steenkamp, D. J., Kenney, W. C., and Singer, T. P. (1978) J. Biol. Chem. 253, 2812–2817
4. Steenkamp, D. J., McIntire, W. S., and Kenney, W. C. (1978) J. Biol. Chem. 253, 2818–2828
5. Hill, C. L., Steenkamp, D. J., Holm, B. H., and Singer, T. P. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 547–551
6. Kasprzak, A. A., Papas, E. J., and Steenkamp, D. J. (1983) Biochem. J. 211, 535–541
7. Lim, L. W., Mathews, F. S., and Steenkamp, D. J. (1988) J. Biol. Chem. 263, 3075–3078
8. Steenkamp, D. J., and Gallup, M. (1978) J. Biol. Chem. 253, 4086–4089
9. Duplessis, E. R., Rohls, R. J., Hille, R., and Thorpe, C. (1994) Biochem. Mol. Biol. Int. 32, 195–199
10. Steenkamp, D. J., Singer, T. P., and Beinert, H. (1978) Biochem. J. 169, 361–369
11. Steenkamp, D. J., Beinert, H., McIntire, W. S., and Singer, T. P. (1978) in Mechanisms of Oxidizing Enzymes (Singer, T. P., and Ondarza, R. N., eds) pp. 127–141, Elsevier North-Holland Inc., New York
12. Singer, T. P., Steenkamp, D. J., Kenney, W. I. C., and Beinert, H. (1980) in Flavins and Flavoproteins (Yagi, K., and Yamano, T., eds) pp. 277–287, Japan Scientific Societies Press, Tokyo
13. Steenkamp, D. J., and Beinert, H. (1982) Biochem. J. 207, 233–239
14. Steenkamp, D. J., and Beinert, H. (1982) Biochem. J. 207, 241–252
15. Rohls, R. J., and Hille, R. (1991) J. Biol. Chem. 266, 15244–15252
16. Rohls, R. J., Huang, L., and Hille, R. (1995) J. Biol. Chem. 270, 22196–22207
17. Huang, L., Rohls, R. J., and Hille, R. (1995) J. Biol. Chem. 270, 23858–23865
18. Rohls, R. J., and Hille, R. (1994) J. Biol. Chem. 269, 30869–30879
19. Falzon, L., and Davidson, V. L. (1996) Biochemistry 35, 2445–2452
20. Strickland, S., Palmer, G., and Massey, V. (1975) J. Biol. Chem. 250, 4048–4052
21. Colly, J., and Zatman, L. J. (1974) Biochem. J. 143, 555–567
22. Armstrong, J. M. (1964) Biochim. Biophys. Acta 86, 194–197
23. Weast, R. C. (ed) Handbook of Chemistry and Physics (1968) 48th Ed., p. D-89, The Chemical Rubber Co., Cleveland, OH.
24. Lim, L. W., Shamala, N., Mathews, F. S., Steenkamp, D. J., Hamlin, R., and Xuong, N. H. (1986) J. Biol. Chem. 261, 15140–15146
25. Baarán, J., Miews, M., Mathews, F. S., and Scrutton, N. S. (1997) Biochem- istry 36, 1989–1998
26. Silverman, R. B. (1995) Accts. Chem. Res. 28, 335–342
27. Edmondson, D. E. (1995) Xenobiotica 25, 735–753
28. Kim, J.-M., Bogdon, M. A., and Mariano, P. S. (1993) J. Am. Chem. Soc. 115, 10591–10595
29. Steenkamp, D. J. (1985) Biochim. Biophys. Res. Commun. 132, 352–359
30. Steenkovich, M. T., and Steenkamp, D. J. (1987) in Flavins and Flavoproteins (Edmonson, D. E., and McCormick, D. B., eds) pp. 127–141, Walter de Gruyter Co., Berlin
31. Hille, R., and Stewart, R. (1984) J. Biol. Chem. 259, 1570–1576
32. Bollamy, H. D., Lim, L. W., Mathews, F. S., and Dunham, W. H. (1989) J. Biol. Chem. 264, 11887–11892
33. Baarán, J., Jang, M-H., Sutcliffe, M. J., Hille, R., and Scrutton, N. S. (1999) J. Biol. Chem. 274, 13155–13161

9 Kinetic studies performed on recombinant TMADH have shown that the obtained rate constants for the fast phase in the reductive half-reaction of TMADH with trimethylamine are comparable to those reported here (the experiments on recombinant protein were performed at 5 °C over a pH range from 6.0 to 9.0, the extrapolated $k_{cat}$ value for the fast phase is greater than 600 s⁻¹ at pH 9.0). The fact that recombinant enzyme from an E. coli expression system and native enzyme obtained from M. methylotrrophus W₆A₆ grown on trimethylamine as carbon source exhibit comparable kinetic behavior suggests that the latter protein has not been modified, as the former almost certainly has not been.