Selective Modification of Alkylammonium Ion Specificity in Trimethylamine Dehydrogenase by the Rational Engineering of Cation-π Bonding[†]

Jaswir Basran,[‡] Martin Mewies,[‡] F. Scott Mathews,[§] and Nigel S. Scrutton*[,‡]

Department of Biochemistry, University of Leicester, Adrian Building, University Road, Leicester LE1 7RH, U.K., and Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

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ABSTRACT: In trimethylamine dehydrogenase (TMADH), substrate is bound in the active site by organic cation-π bonding mediated by residues Tyr-60, Trp-264, and Trp-355. In the closely related dimethylamine dehydrogenase (DMADH), modeling suggests that a mixture of cation-π bonding and conventional hydrogen bonding is responsible for binding dimethylamine. The active sites of both enzymes are highly conserved, but three changes in amino acid identity (residues Tyr-60 → Gln, Ser-74 → Thr, and Trp-105 → Phe, TMADH numbering) were identified as probable determinants for tertiary → secondary alkylammonium ion specificity. In an attempt to switch the substrate specificity of TMADH so that the enzyme operates more efficiently with dimethylamine, three mutant proteins of TMADH were isolated. The mutant forms contained either a single mutation (Y60Q), double mutation (Y60Q·S74T) or triple mutation (Y60Q·S74T·W105F). A kinetic analysis in the steady state with trimethylamine and dimethylamine as substrate indicated that the specificity of the triple mutant was switched approximately 90 000-fold in favor of dimethylamine. The major component of this switch in specificity is a selective impairment of the catalytic efficiency of the enzyme with trimethylamine. Rapid-scanning and single wavelength stopped-flow spectroscopic studies revealed that the major effects of the mutations are on the rate of flavin reduction and the dissociation constant for substrate when trimethylamine is used as substrate. With dimethylamine as substrate, the rate constants for flavin reduction and the dissociation constants for substrate are not substantially affected in the mutant enzymes compared with wild-type TMADH. The results indicate a *selective* modification of the substrate-binding site in TMADH (that impairs catalysis with trimethylamine but not with dimethylamine) is responsible for the switch in substrate specificity displayed by the mutant enzymes.

Cation-π bonding is now established as an important contributor to the molecular architecture of proteins (Burley & Petsko, 1986; Mitchell et al., 1994; Flocco & Mowbray, 1994). More recently, however, its role in protein−ligand interactions has also been appreciated (Dougherty, 1996; Scrutton & Raine, 1996). Cation-π bonding in protein−ligand complexes has been observed in molecules as functionally and structurally diverse as acetylcholinesterase (Sussman et al., 1991), the McPC603 Fab mouse myeloma IgA (κ) (Davis & Metzger, 1983; Satow et al., 1986), and the SH2 domain of the v-*src* oncogene (Waksman et al., 1992). The alkylammonium ion forms the largest group of ligand molecules containing organic cations that are of direct relevance to biology. These molecules are widespread; they include the large and growing family of synthetic therapeutic and abused drugs, several physiological amines (for example, histamine, catecholamines, and acetylcholine), and peptides and "protein elements" involved in a variety of signaling mechanisms. In recent years, there has been a steady growth in the number of determined protein structures that form complexes with substituted ammonium ligands, and the majority of these protein−ligand interactions employ cation-π bonding (Scrutton & Raine, 1996). Consequently, a comprehensive understanding of cation-π bonding will impact directly on structure-based drug design, and with this in mind, we have developed trimethylamine dehydrogenase (TMADH)[1] as a simple model to investigate the determinants of alkylammonium ion specificity.

TMADH (EC 1.5.99.7) is an iron−sulfur flavoprotein that catalyzes the oxidative demethylation of trimethylamine to produce dimethylamine and formaldehyde (Steenkamp & Mallinson, 1976).

$$(CH_3)_3N + H_2O \rightarrow (CH_3)_2NH + CH_2O + 2e^- + 2H^+$$

The enzyme is found in methylotrophic bacteria where it enables the organism to subsist on trimethylamine as the sole source of carbon. Electrons derived from the oxidation of trimethylamine are transferred first to a 6-*S*-cysteinyl-FMN in TMADH and then sequentially to the 4Fe-4S center of the enzyme. An electron-transferring flavoprotein (ETF; Steenkamp & Gallup, 1978; Chen & Swenson, 1994) that associates with TMADH as part of the reaction cycle is responsible for accepting the electrons from TMADH during the oxidative half-reaction. Due in part to the availability of the cloned gene encoding TMADH (Boyd et al., 1992) and a high-resolution crystal structure of the enzyme (Lim

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* Corresponding author: telephone, +44 116 223 1337; telefax, +44 116 252 3369; email, nss4@le.ac.uk.

[‡] University of Leicester.

[§] Washington University School of Medicine.

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[1] Abbreviations: TMADH, trimethylamine dehydrogenase; DMADH, dimethylamine dehydrogenase.

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et al., 1986; F. S. Mathews et al., unpublished), the reaction catalyzed by TMADH has been studied intensively by stopped-flow methods and protein engineering. Rate constants for electron transfer from the iron−sulfur center of TMADH to the flavin of ETF have been determined (Huang et al., 1995; Wilson et al., 1997a), and residues on the surface of TMADH that mediate electron transfer to ETF have been identified (Wilson et al., 1995, 1997a,b). In the absence of substrate, the kinetics of internal electron transfer from the 6-*S*-cysteinyl-FMN to the 4Fe-4S center have also been investigated by pH-jump stopped-flow methods (Rohlfs & Hille, 1991; Rohlfs et al., 1995). The reductive half-reaction with the slow substrate diethylmethylamine (*viz.* reduction of the 6-*S*-cysteinyl-FMN by substrate followed by internal electron transfer) occurs in three kinetically resolvable phases: a fast phase attributed to reduction of the flavin, an intermediate phase reflecting internal electron transfer and the breakdown of the putative substrate−flavin intermediate, and a slow phase that describes the formation of a spin-interacting state (Rohlfs & Hille, 1994). With trimethylamine as substrate, the intermediate and slow phases of the reductive half-reaction are dependent on substrate concentration, which may reflect gating of internal electron transfer (Falzon & Davidson, 1996a). These detailed kinetic experiments provide an excellent framework to explore by protein engineering techniques the molecular recognition by TMADH of native and foreign alkylammonium substrates.

Previous and on-going work on crystalline TMADH soaked in the presence of the substrate inhibitor tetramethylammonium chloride demonstrated that these compounds are accommodated by cation-π bonding in an "aromatic bowl" of three residues (Tyr-60, Trp-264, and Trp-355) at the active site of the enzyme (Bellamy et al., 1989; F. S. Mathews et al., unpublished). Trimethylamine is the simplest tertiary alkylammonium ion, and therefore, the interaction observed in the crystal structure of TMADH is an excellent model for studying organic cation−protein interactions. The simplest secondary alkylammonium ion, dimethylammonium, is bound by the related enzyme dimethylamine dehydrogenase (DMADH) from *Hyphomicrobium X*, which shares 63.5% sequence identity with TMADH (Yang et al., 1995).[2] The high degree of sequence identity between the two enzymes enabled us to construct a model for DMADH (Raine et al., 1995). Close examination of this model suggests differences in the active site regions of these proteins that may account for the exclusivity in substrate specificity displayed by each enzyme. In the study reported here, the molecular model of DMADH has been analyzed to identify changes in active site architecture that may favor the binding of the secondary alkylammonium (dimethylamine) over the tertiary alkylammonium (trimethylamine) substrate. Selected changes in the active site have been introduced into the TMADH catalytic framework by conventional site-directed mutagenesis. Stopped-flow kinetic analyses of the reductive half-reaction in wild-type and mutant forms of TMADH are used to demonstrate that, in certain mutant proteins, the specificity of the enzyme is switched in favor of the secondary alkylammonium substrate. In the main, the switch in specificity is the result of a selective impairment in the specificity for the trimethylammonium cation with only a small perturbation in the specificity seen for dimethylammonium. The result is surprising, in light of the fact that the substrate-binding sites are common for the secondary and tertiary ammonium substrates.

[2] Dimethylamine is a poor substrate for *Methylophilus methylotrophus* (sp. W_3A_1) TMADH. *Hyphomicrobium X* contains both a dimethylamine dehydrogenase and a trimethylamine dehydrogenase, and each is highly specific for its own substrate.

MATERIALS AND METHODS

Isolation of Mutant TMADH Enzymes. General methods used for recombinant DNA work were adopted from Sambrook et al. (1989). Site-directed mutagenesis was performed using the Unique Site Elimination (U.S.E.) mutagenesis kit supplied by Pharmacia. The expression plasmid for wild-type TMADH (pSV2tmdveg; Scrutton et al., 1994) was used as template DNA for the isolation of the Y60Q mutant TMADH (mutagenic oligonucleotide 5′-GGTTAATGGAG-CATTGTTCAGTGT-3′), and the selection primer (5′-CGGATCTCGTGCCATGAACTCCACCTCACTAC-3′) was used to eliminate the *Eco*RI site found immediately upstream of the *tmd* gene (Scrutton et al., 1994). Mutant plasmids were identified by restriction analysis and dideoxy DNA sequencing (Sanger & Coulson, 1975). To minimize the amount of resequencing (performed to ensure no spurious changes arose during the mutagenesis procedure) and to restore the upstream *Eco*RI site for further rounds of mutagenesis (see below), a 1.4 kbp *Kpn*I DNA fragment containing approximately 50% of the *tmd* gene was excised from the mutagenized plasmid and used to replace the equivalent fragment in plasmid pSV2tmdveg. The new construct was designated pSV2tmdvegY60Q. The double (Y60Q·S74T) and triple (Y60Q·S74T·W105F) mutant forms of TMADH were isolated from the same mutagenesis reaction using plasmid pSV2tmdvegY60Q as template DNA and the following mutagenic oligonucleotides: 5′-CCA-GATACGTGCGGTCAAACGATG-3′ (S74T) and 5′-G-CACCGCCGTAGAATAACTCGACC-3′ (W105F). Both mutants were identified by restriction analysis and DNA sequencing, and they were subsequently subcloned into plasmid pSV2tmdveg as described above. Each mutant form of TMADH was purified to homogeneity as described previously (Scrutton et al., 1994), and subunit concentrations were determined spectrophotometrically at 280 nm ($\epsilon = 201,610\ M^{-1}\ cm^{-1}$). The *Escherichia coli* strain transformed with the triple mutant was grown at 20 °C to suppress the formation of inclusion bodies. Cofactor stoichiometries for the recombinant enzyme samples were determined spectrophotometrically as described previously (Scrutton et al., 1994; Mewies et al., 1996). Wild-type TMADH was purified from *Methylophilus methylotrophus* (W_3A_1) as described by Steenkamp and Mallinson (1976), incorporating the modifications of Wilson et al. (1995). The concentration of wild-type TMADH subunit was determined spectrophotometrically at 443 nm ($\epsilon_{443} = 27\,300\ M^{-1}\ cm^{-1}$). All chemicals were of analytical grade wherever possible. Glass-distilled water was used throughout.

Steady-State and Stopped-Flow Kinetic Analyses. Steady-state kinetic measurements were performed with a 1 cm light path in a final volume of 1 mL. The desired concentrations of trimethylamine, dimethylamine, phenazine methosulfate (PMS), and 2,6-dichlorophenolindophenol (DCPIP) were obtained by making microliter additions from stock solutions to the assay mix. Assays for the determination of kinetic parameters were performed in 100 mM sodium pyrophos-

phate buffer, pH 8.5. Reactions were started by the addition of substrate, and the decrease in absorption at 600 nm due to reduction of DCPIP ($\epsilon = 21\,900\ M^{-1}\ cm^{-1}$) was measured using a Hewlett-Packard 8452A single-beam diode array spectrophotometer. All data were collected at 30 °C. Data were fitted to the appropriate rate equation (see Results) using the fitting program Kaleidograph (Abelbeck Software, Reading, PA).

Rapid reaction kinetic experiments were performed using an Applied Photophysics SF.17MV stopped-flow spectrophotometer. Time-dependent aerobic reductions of TMADH with dimethylamine and trimethylamine at pH 7.5 were performed by rapid-scanning stopped-flow spectroscopy using a photodiode array detector and X-SCAN software (Applied Photophysics). Spectral deconvolution was perfomed by global analysis and numerical integration methods using PROKIN software (Applied Photophysics). For single wavelength studies, data collected at 443 nm were analyzed using nonlinear least squares regression analysis on an Archimedes 410-1 microcomputer using Spectrakinetics software (Applied Photophysics). Experiments were performed by mixing TMADH in 100 mM potassium phosphate buffer, pH 7.5, with an equal volume of trimethylamine or dimethylamine contained in the same buffer at the desired concentration. In stopped-flow experiments, the concentration of substrate was always at least 10-fold greater than that of TMADH, thereby ensuring pseudo-first-order conditions. For each substrate concentration, at least five replica measurements were collected. Previously, we demonstrated that substrate-reduced TMADH is quite stable to reoxidation in aerobic environments (half-life about 50 min; Wilson et al., 1995). Consequently, the need to use anaerobic conditions in the stopped-flow experiments was negated.

As previously noted by other workers (Rohlfs & Hille, 1995; Falzon & Davidson, 1996a), under pseudo-first-order conditions, the observed rate constant (k_{obs1}) for the absorbance change at 443 nm (flavin reduction) is obtained from fits of the data to the equation for a single exponential decay. At 443 nm, the rate constants were found to depend on substrate concentration, and the reaction sequence was modeled as shown in the general scheme of the equation

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C \underset{k4}{\overset{k_3}{\rightleftharpoons}} D \qquad (1)$$

where A = $TMADH_{ox}$, B = substrate, C = Michaelis complex, and D = TMADH containing the dihydroflavin form of the S^6-cysteinyl-FMN (Falzon & Davidson, 1996a). Data for the mutant enzymes and the wild-type enzyme with dimethylamine as substrate were fitted to the equation (Strickland et al., 1975)

$$k_{obs} = \frac{k_3[S]}{K_d + [S]} + k_4 \qquad (2)$$

As noted by Falzon and Davidson (1996a), the wild-type enzyme does not show hyperbolic dependence on trimethylamine concentration since $k_1[S] + k_2 \gg k_3 + k_4$. In this instance, fitting was to the equation derived by Hiromi (Hiromi, 1979; Falzon & Davidson, 1996a). All curve fitting was performed using the Kaleidograph software package (Abelbeck Software, Reading, PA).

The absorbance changes at 365 nm were biphasic for wild-type enzyme with trimethylamine, and k_{obs2} and k_{obs3} were assigned as the observed rate constants for the faster and slower phases, respectively. The observed rate constants k_{obs2} and k_{obs3} were found to depend on trimethylamine concentration, as reported previously (Falzon & Davidson, 1996a) and were analyzed independently by fitting to eq 2. With dimethylamine as substrate, the absorbance changes at 365 nm for the wild-type protein were either biphasic or monophasic, and the rate constants were dependent on substrate concentration. For all the mutant proteins with trimethylamine and dimethylamine as substrate, absorbance changes at 365 nm were also dependent on substrate concentration but were monophasic (but see comments concerning the Y60Q mutant in Results). For these mutants, limiting rate constants and dissociation constants were calculated by fitting the values of k_{obs} to eq 2.

RESULTS

Modeling of Alkylammonium Specificity in DMADH and TMADH. In previous work, we cloned and sequenced the gene (*dmd*) encoding dimethylamine dehydrogenase from *Hyphomicrobium X* and demonstrated that the gene is 63.5% identical in sequence to the gene (*tmd*) encoding trimethylamine dehydrogenase (Yang et al., 1995). The high sequence identity of the two proteins enabled a model for the structure of DMADH to be constructed using the crystal coordinates of TMADH (Raine et al., 1995). Within the active site regions of the DMADH model and the crystal structure of TMADH, the identity of amino acids was found to be almost perfect. All the residues involved in the chemistry of demethylation are totally conserved, *viz*., the cysteine residue that forms the 6-*S*-cysteinyl-FMN, required to prevent adventitious attack of the flavin by solvent (Huang et al., 1996), the histidine required to breakdown a covalent intermediate formed in catalysis (Rohlfs & Hille, 1994), and an arginine that stabilizes negative charge as it develops in the region of the N1 atom and C2 carbonyl during catalysis and flavinylation of the enzyme (Mewies et al., 1996). The active site base has not yet been formally assigned, but all candidate residues are conserved in DMADH. The only striking difference between the two proteins is found in the substrate-binding aromatic bowl comprising residues Tyr-60, Trp-264, and Trp-355; these residues form cation-π bonds with the inhibitor tetramethylammonium ion in TMADH. In DMADH, the residue equivalent to Tyr-60 of TMADH is glutamine, and we demonstrated previously by modeling that the $O_{\epsilon 1}$ of this glutamine residue may form a conventional hydrogen bond with the N−H of the substrate dimethylammonium (Raine et al., 1995). Beyond the first neighbor shell for substrate, we have also identified two further and previously unreported differences between the two enzymes that may contribute to the exclusivity in alkylammonium ion specificity. These residues in TMADH are Ser-74 and Trp-105 which pack against Tyr-60 of the substrate-binding aromatic bowl. In DMADH, these residues are exchanged for Thr-74 and Phe-105. In our energy-minimized model of DMADH (Raine et al., 1995), these residues appear to influence the positioning of Gln-60 so that the $O_{\epsilon 1}$ of this residue is capable of forming a good hydrogen bond with the N−H of dimethylammonium (Figure 1).

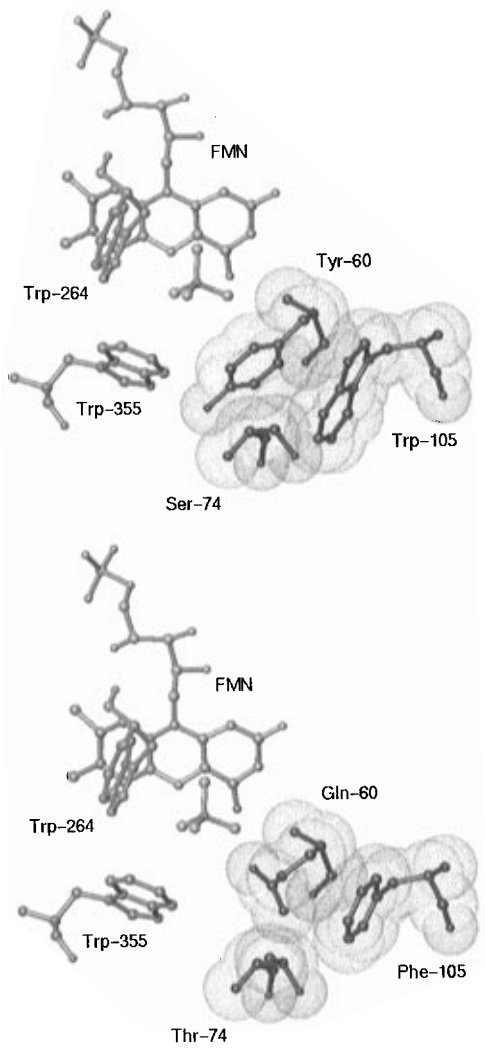

FIGURE 1: Molecular graphics representation of the active site of (a, top) trimethylamine dehydrogenase and the model of (b, bottom) dimethylamine dehydrogenase. Details of the model construction are given in Raine et al. (1995). A van der Waals surface is displayed around the three residues that are not conserved in the active sites of DMADH and TMADH and which are thought to account for the difference in substrate specificity. This diagram was prepared using the molecular graphics program TURBO-FRODO (Roussel & Cambillau, 1991).

Our modeling work suggests that it might be possible to switch the alkylammonium ion specificity of TMADH by exchanging residues Tyr-60, Ser-74, and Trp-105 for their counterparts found in DMADH. To test this assertion, we isolated three mutant forms of TMADH: in the first, Tyr-60 was exchanged for glutamine (mutant Y60Q). The Ser-74 → Thr and Trp-105 → Phe substitutions were then introduced into the Y60Q mutant in a stepwise manner to produce the double mutant (Y60Q·S74T) and the triple mutant (Y60Q·S74T·W105F) forms of TMADH.

General Properties of the Mutant Enzymes. The three mutant forms of TMADH were purified as described below. Using previously published methods (Scrutton et al., 1994), the iron−sulfur center and ADP cofactors were found to be stoichiometrically assembled with each mutant enzyme. The proportion of flavinylated enzyme in each preparation was variable (range 20−30% of total enzyme sample) but consistent with previous published data for recombinant wild-type (Scrutton et al., 1994) and selected mutant forms of TMADH [e.g., Wilson et al. (1997a)]. The reasons for obtaining partially flavinylated recombinant material in purified enzyme samples have been discussed elsewhere (Mewies et al., 1996; Mathews et al., 1996). The results of a steady-state kinetic analysis for each mutant TMADH, performed at the optimum pH for activity (pH 8.5) and using trimethylamine and dimethylamine as substrate, are displayed in Table 1. Data were fitted to eq 3 (Falzon & Davidson, 1996a) for the wild-type and Y60Q enzymes with trimethylamine as substrate; this equation takes into account the observed substrate inhibition thought to be caused by the binding of a second molecule of trimethylamine to the enzyme.

Table 1: Steady-State Kinetic Parameters for the Wild-Type and Mutant Forms of TMADH with Trimethylamine and Dimethylamine as Substrate

	trimethylamine		
enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
wild type[a]	13.7 ± 1.7	15.6 ± 2.4	1.14×10^6
Y60Q[a]	545 ± 280	0.08 ± 0.01	147
Y60Q·S74T	737 ± 71	0.13 ± 0.004	176
Y60Q·S74T·W105F	8740 ± 2700	0.02 ± 0.003	2.29
	dimethylamine		
enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
wild type	2.34 ± 0.19	0.67 ± 0.02	286
Y60Q	2.52 ± 0.47	0.55 ± 0.03	218
Y60Q·S74T	3.12 ± 0.45	0.32 ± 0.015	102
Y60Q·S74T·W105F	7.25 ± 0.88	0.38 ± 0.024	52

[a] Data calculated by fitting to eq 3. $K_i = 1 \pm 0.3$ mM, $b = 0.24 \pm 0.03$ for the wild-type enzyme and $K_i = 2.6 \pm 2.4$ mM, $b = 0.32 \pm 0.1$ for the Y60Q mutant enzyme. k_{cat} values for the wild-type and Y60Q enzymes with trimethylamine as substrate were calculated from the observed V_{max} ($bk_p[E_T]$) rather than the theoretical V_{max} ($k_p[E_T]$) that would have been seen in the absence of substrate inhibition (Falzon & Davidson, 1996a). All other data were calculated by fitting to the standard Michaelis−Menten equation, since no substrate inhibition was apparent over the concentration range used in the assays (i.e., up to 25 mM).

$$v = \frac{V_{max}\left(\frac{1}{b} + \frac{[S]}{K_i}\right)}{1 + \frac{K_S}{[S]} + \frac{K_S}{K_i} + \frac{[S]}{K_i}} \quad (3)$$

In eq 3, b is a factor that affects the intrinsic maximum velocity; all other parameters have their usual meaning. Substrate inhibition at pH 8.5 ($K_i = 1 \pm 0.3$ and $K_i = 2.6 \pm 2.4$ mM for the wild-type and Y60Q mutant enzymes, respectively) is less pronounced than that observed at pH 7.5 ($K_i = 52 \pm 15$ μM for the wild-type enzyme), the latter value being the one reported by Falzon and Davidson (1996a) in their analysis of the wild-type enzyme. Neither the double (Y60Q·S74T) nor triple (Y60Q·S74T·W105F) mutant enzymes displayed substrate inhibition with trimethylamine as substrate over the concentration range used (up to 25 mM). Similarly, in the steady-state assays of the wild-type and mutant enzymes with dimethylamine, no substrate inhibition was observed in the concentration range used (up to 25 mM). In the absence of substrate inhibition, data were best fitted to the standard Michaelis−Menten equation.

From the steady-state data, it is apparent that dimethylamine is a substrate for wild-type TMADH, albeit a poor one [ratio of selectivity coefficients (i.e., k_{cat}/K_m) for TMA

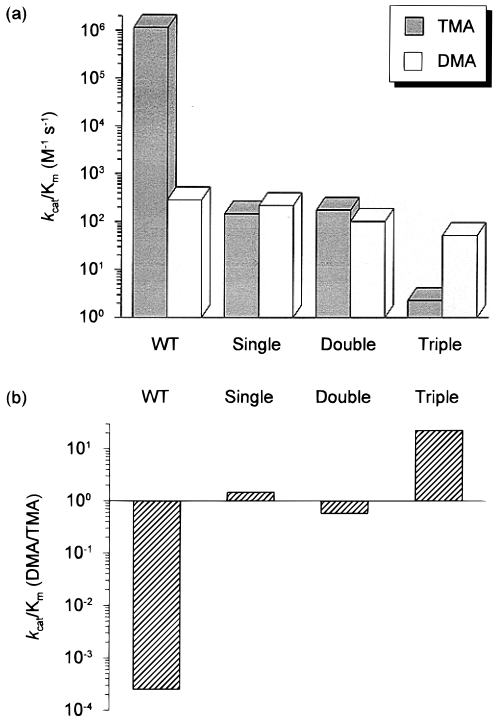

FIGURE 2: Histograms illustrating (a) the effects of mutations on the selectivity coefficient for trimethylamine and dimethylamine and (b) the magnitude of the switch in substrate specificity. Key: WT, wild-type enzyme; single, mutant Y60Q; double, mutant Y60Q·S74T; triple, mutant Y60Q·S74T·W105F.

and DMA is about 4000]. The steady-state reaction of wild-type TMADH with DMA is compromised mainly by a poor Michaelis constant for the substrate (2.3 mM compared with 13.7 μM for TMA), although the value for k_{cat} also contributes to some extent (0.67 s^{-1} compared with 15.6 s^{-1} for TMA). Mutation of Tyr-60 to glutamine leads to a large increase in the Michaelis constant for TMA, and this is raised further by the introduction of a Thr-74 and Phe-105 in the double and triple mutant forms of TMADH. Concomitantly, there is about a 100-fold fall in k_{cat} for the Y60Q and Y60Q·S74T mutant enzymes and a 800-fold fall for the Y60Q·S74T·W105F triple mutant. The data are consistent with Tyr-60 being an important determinant for the binding of trimethylamine in the active site of the wild-type enzyme: inappropriate positioning of the substrate through mutational changes are expected to have effects on both turnover and the Michaelis constant for trimethylamine.

Following mutagenesis, only very modest changes were observed in the measured steady-state kinetic parameters using dimethylamine as substrate. No large increases in K_m or decreases in k_{cat} were observed throughout the series of enzymes. The result is perhaps surprising given that trimethylamine and dimethylamine bind at the same location in the active site. In the steady state, therefore, the major effect of mutation is to *selectively* impair catalysis observed with trimethylamine while only slightly compromising the level of dimethylamine-dependent activity seen in the wild-type enzyme. The magnitude of the switch in specificity [ratio of k_{cat}/K_m (DMA/TMA) for Y60Q·S74T·W105F and k_{cat}/K_m (DMA/TMA) for wild type] is 90 000-fold (Figure 2). However, analysis of the steady-state behavior of the mutant enzymes alone does not report on any effects the mutations may have in the reductive half-reaction, i.e., dissociation constant for substrates and rates of flavin reduction. For this reason, we also performed an analysis of the reductive half-reaction of the wild-type and mutant proteins by stopped-flow spectroscopy.

The Reductive Half-Reaction: Intermediates and Spectral Analysis. Wild-Type TMADH with Trimethylamine and Dimethylamine at pH 7.5. In the single wavelength stopped-flow studies of the reductive half-reaction (see below), reactions were performed at pH 7.5. In previous work on the reductive half-reaction using the slow substrate diethylmethylamine (Rohlfs & Hille, 1994), the fast phase of the reaction (reduction of flavin) and the dissociation constant for the substrate were found to be pH-dependent: flavin reduction requires the basic form of a group within the enzyme−substrate complex with pK_a 7.9, and for maximum catalytic activity, the basic form of an ionizable group within the free enzyme displaying a pK_a value of 8.7 is required (Rohlfs & Hille, 1994). Although pH 7.5 is perhaps the most convenient pH value to examine the kinetics of the reductive half-reaction (especially in terms of slowing the rate of flavin reduction in the wild-type enzyme with trimethylamine; see below), maximal rates for enzyme reduction are observed at pH 10. However, flavin reduction rates are not accessible by stopped-flow spectroscopy at pH 10, and rates for the wild-type and mutant enzymes were therefore measured at pH 7.5 where they are readily observed. As a prerequisite to performing single wavelength stopped-flow reactions, we have examined the reductive half-reaction of wild-type and mutant forms of TMADH using trimethylamine and dimethylamine as substrate.

At pH 7.5, spectral changes accompanying the reduction of TMADH by trimethylamine have been reported previously [e.g., Falzon and Davidson (1996a)], although the individual spectra for reaction intermediates in the reductive half-reaction with this substrate were not determined. Using difference spectroscopy techniques, the spectra of intermediates formed during the reaction of TMADH with the slow substrate diethylmethylamine have been calculated (Rohlfs & Hille, 1994). In the work reported here, and as a guide to single wavelength studies (see below), we have resolved the spectra of intermediates formed during the reaction of wild-type and mutant TMADH enzymes with dimethylamine and trimethylamine using global analysis and numerical integration techniques. For the wild-type enzyme with trimethylamine as reductant, the time-dependent absorbance changes and the spectral forms of intermediates are displayed in Figure 3. In this case, the spectral forms of the intermediates are similar to those calculated by difference spectroscopy in studies of the reductive half-reaction employing diethylmethylamine as substrate (Rohlfs & Hille, 1994), although, in light of recent work (Falzon & Davidson, 1996a), we interpret the identity of selected intermediates differently. Spectral deconvolution by global analysis and numerical integration was performed by fitting to the model in the scheme

$$A \rightarrow B \rightarrow C \rightarrow D$$

where A = oxidized enzyme, B = reduced enzyme (dihydroflavin), C = mixture of two forms of reduced enzyme (a proportion in the dihydroflavin form mixed with a proportion in the semiquinone/reduced iron−sulfur center form), and D = reduced enzyme (semiquinone/reduced iron−sulfur center). Form C arises because internal electron transfer

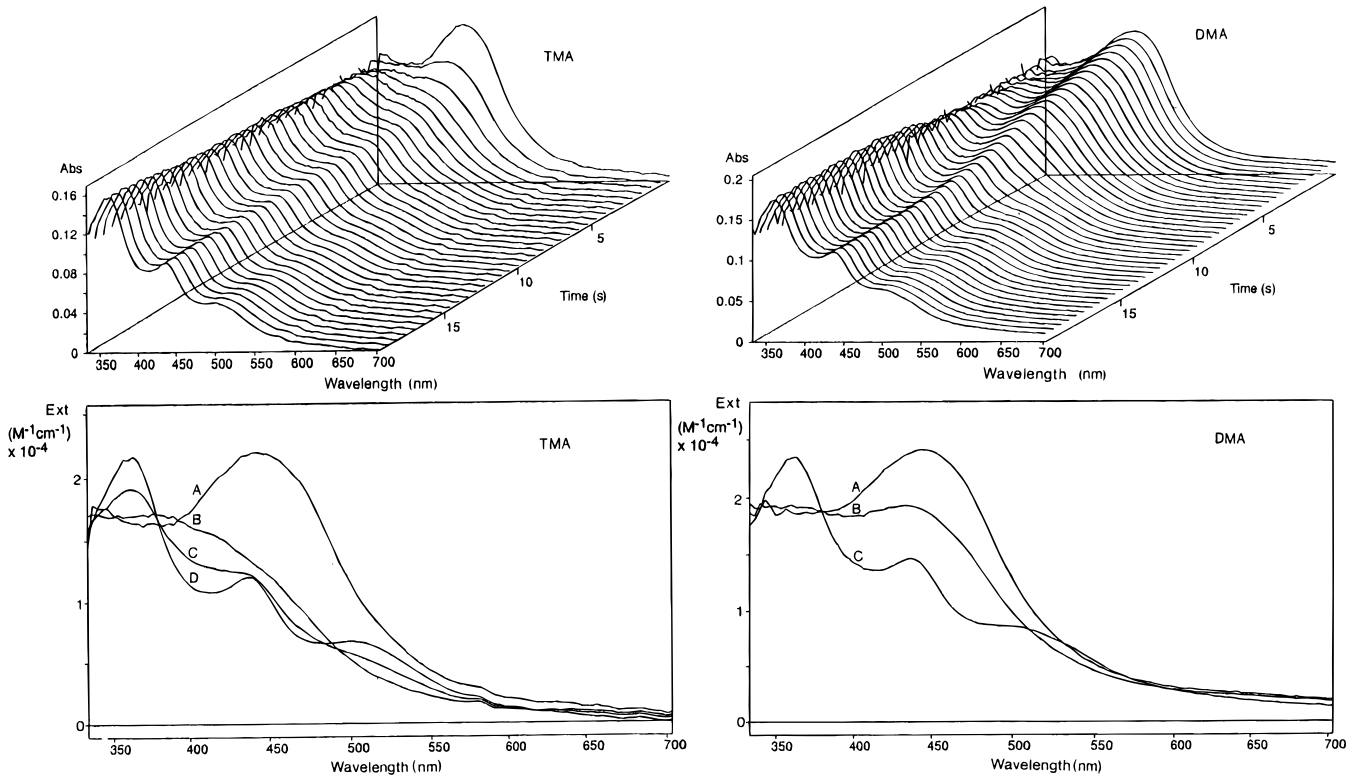

FIGURE 3: Time-dependent spectral changes for the wild-type enzyme with trimethylamine and dimethylamine as substrate and deconvoluted spectra for the reaction intermediates. (a, left) 7.5 μM wild-type TMADH mixed with 125 μM trimethylamine at 30 °C, pH 7.5. (b, right) 7.5 μM wild-type TMADH mixed with 20 mM dimethylamine at 30 °C, pH 7.5. With trimethylamine, A = oxidized enzyme, B = reduced enzyme (dihydroflavin), C = mixture of two forms of reduced enzyme (a proportion in the dihydroflavin form mixed with a proportion in the semiquinone/reduced iron−sulfur center form), and D = reduced enzyme (semiquinone/reduced iron−sulfur center); for details see text. With dimethylamine, A = oxidized enzyme, B = a spectral and not true kinetic intermediate which appears because of kinetic mixing of the 443 nm phase with the 365 nm phase (see text), and C = reduced enzyme (semiquinone/reduced iron−sulfur center).

from the flavin to the iron−sulfur center in a subpopulation of enzymes is retarded through gating by the binding of a second molecule of substrate (Falzon & Davidson, 1996a), and therefore, a proportion of the enzyme will be present in the dihydroflavin form: those enzyme molecules that lack the second (modulating) substrate in the active site and therefore escape gating will transfer an electron from the dihydroflavin to yield the "semiquinone/reduced iron−sulfur center" form of the enzyme. Form C is therefore not a true enzyme intermediate but is a spectral intermediate that is contributed by two forms of the enzyme. Following gated electron transfer, intermediate D is formed, which is the enzyme species formed at the end of the reductive half-reaction. From the spectral forms of the four discrete states (Figure 3), it can be seen that the absorbance change at 443 nm during the reductive half-reaction predominantly (>95% signal) represents reduction of the flavin to produce the dihydroflavin (species B) form of TMADH, and changes at 365 nm are associated with the formation of species C and D (i.e., reporting on internal electron transfer from dihydroflavin to the iron−sulfur center). These two wavelengths were therefore selected for the single wavelength studies described below.

Rapid-scanning spectroscopy methods were also performed with the wild-type enzyme at pH 7.5 using dimethylamine as substrate. In this case, however, data could not be fitted to the four-state reductive half-reaction scheme described above; spectral deconvolution yielded inappropriate intermediate and starting spectra, and the model was at variance with much of the kinetic data. The data were best fit using the scheme

$$A \rightarrow B \rightarrow C$$

where A represents oxidized enzyme and C is the equivalent of species D during reduction with trimethylamine, i.e., the enzyme species present at the end of the reductive half-reaction (Figure 3). In terms of the spectral form, species C (dimethylamine) is very similar to species D (trimethylamine). Species B (dimethylamine) is distinct from both species C (trimethylamine) and species D (trimethylamine) and, in fact, appears to have spectral properties of both the latter intermediates. For example, species B (dimethylamine) has increased (albeit small) absorption at 365 nm and decreased absorption at 443 nm. The deconvoluted spectra suggest that reduction of the flavin (changes at 443 nm) and the faster of the two phases occurring at 365 nm (internal electron transfer) are not resolved kinetically in the rapid-scanning experiment, and as such species B (dimethylamine) is not a true intermediate in the reductive half-reaction. This suggestion is highly plausible, given the much slower rate of reduction of the wild-type enzyme with dimethylamine compared with trimethylamine (see single wavelength data below); kinetic mixing of the 443 and 365 nm phases is clearly seen in the time-dependent spectral changes seen for wild-type TMADH with dimethylamine (Figure 3). Absorption changes occurring during the conversion of B → C (dimethylamine) do not reflect gated internal electron transfer (as noted for C → D with trimethylamine). In this regard, it is important to note that, unlike in the steady state with trimethylamine, there is no apparent substrate inhibition when dimethylamine is used as substrate (see above). Substrate inhibition with trimethylamine has been attributed to gated

Table 2: Limiting Rate Constants and Dissociation Constants Calculated from the Single Wavelength (443 nm) Stopped-Flow Data for Wild-Type and Mutant TMADH Enzymes at pH 7.5

	trimethylamine			dimethylamine		
enzyme	k_{lim} (s^{-1})	K_d (mM)	k_{lim}/K_d (s^{-1} M^{-1})	k_{lim} (s^{-1})	K_d (mM)	k_{lim}/K_d (s^{-1} M^{-1})
wild type[a]	706 ± 15	0.21 ± 0.06	3.36×10^6	19.2 ± 1.2	117 ± 14	164
Y60Q	2.15 ± 0.1	52.5 ± 5.3	40.9	1.13 ± 0.1	100 ± 24	11.3
Y60Q·S74T	1.77 ± 0.1	76.8 ± 12	23.0	1.0 ± 0.1	119 ± 13	8.40
Y60Q·S74T·W105F	0.16 ± 0.01	86.6 ± 9.4	1.85	0.78 ± 0.03	105 ± 7.6	7.42

[a] Data for wild-type TMADH with trimethylamine did not show a hyperbolic dependence on substrate concentration and consequently were fitted using the equation derived by Hiromi (Hiromi, 1979; Falzon & Davidson, 1996a). A larger error in the determined value of K_d is a necessary consequence of fitting to an equation of this complexity, rather than fitting to the simpler Strickland equation. All other data in the table were fitted using the Strickland equation (eq 2).

electron transfer from the flavin to the 4Fe-4S center of TMADH caused by the binding of a second molecule of trimethylamine at the active site (Falzon & Davidson, 1996a). The absorption changes at 365 nm in the reaction B → C (dimethylamine) are associated with nongated reduction of the 4Fe-4S center and the formation of the spin-interacting state at the end of the reductive half-reaction; similar absorption changes at 365 nm have been observed in the reaction of TMADH with the slow substrate diethylmethylamine, and they have been attributed to reduction of the 4Fe-4S center and formation of the spin-interacting state (Rohlfs & Hille, 1994). Further evidence for the mixing of kinetic phases in the reductive half-reaction of wild-type enzyme with dimethylamine is presented in the results of single wavelength studies at 365 nm (see below).

For the mutant proteins reduced with dimethylamine and trimethylamine at pH 7.5, we were also unable to deconvolute the reductive half-reaction in the way described for the wild-type enzyme with trimethylamine. The data were best fit using the second model described for the wild-type enzyme with dimethylamine, and the "intermediate" spectral forms were similar (although not identical due to underflavinylation of the mutant proteins) to those deconvoluted for wild-type TMADH−dimethylamine. Mixing of the kinetic phases for the 443 and 365 nm absorption changes is the most likely cause of this behavior (and see single wavelength studies below).

The rapid-scanning stopped-flow data indicate that 443 nm is an appropriate wavelength to monitor flavin reduction in the wild-type and mutant enzymes. Absorbance changes at 365 nm can also be used to follow internal electron transfer from the flavin to the 4Fe-4S center. However, in those circumstances where the first kinetic phase of the reductive half-reaction is substantially slowed, either by mutation and/or by using dimethylamine as substrate, then mixing of the first and second kinetic phases occurs, and it is not possible to resolve the spectra of true intermediates. When the nongating and slow substrate diethylmethylamine is used above pH 7, the intermediate and slow kinetic phases (measured at 365 nm) of the reductive half-reaction are resolved from the fast phase, and they display no dependence on substrate concentration (Rohlfs & Hille, 1994). Below pH 7, however, the fast phase is sufficiently retarded to become mixed with the intermediate phase, and under these conditions an apparent substrate dependence on the intermediate phase is observed. From the rapid-scanning stopped-flow work, our observations on the wild-type enzyme with dimethylamine and mutant enzymes with either substrate suggest that in single wavelength studies an apparent substrate dependence at 365 nm should be observed due to kinetic mixing of the first and second steps of the reductive half-reaction (see below).

Single Wavelength Stopped-Flow Studies. (A) Single Wavelength Studies at 443 nm. Single wavelength studies at 443 nm were used to measure the rates of flavin reduction in the wild-type and mutant enzymes using trimethylamine and dimethylamine as substrate (Table 2). In all cases, kinetic traces were monophasic and depended on substrate concentration. Data for the wild-type enzyme with trimethylamine as substrate did not show a hyperbolic dependence on substrate concentration, and the data were best fit to the equation derived in Hiromi (1979; see Materials and Methods). The dissociation constant for trimethylamine was, therefore, calculated as the ratio of the microscopic rate constants k_1 and k_2 (0.54 ± 0.03 and 113 ± 30 s^{-1}, respectively) that were determined from the fitting procedure. A hyperbolic dependence on substrate concentration was found for all enzymes using dimethylamine as substrate and each mutant enzyme operating with trimethylamine as substrate.

With trimethylamine as reductant, the data revealed that each mutant enzyme has an increased dissociation constant for substrate (by about 300-fold) when compared with the wild-type enzyme. The rate of flavin reduction is severely compromised in each of the mutant forms and is most marked for the Y60Q·S74T·W105F mutant enzyme (>4000-fold reduction in rate constant). No effect on the dissociation constant for dimethylamine was seen in any of the mutant enzymes when compared with the wild-type protein, and only a small decrease in the rate of flavin reduction was observed (about 25-fold reduction for the Y60Q·S74T·W105F mutant enzyme). Values of k_{lim}/K_d for both substrates and each enzyme (Table 2) are plotted in Figure 4, and the trends were similar to those observed in the steady-state kinetic analysis of the enzymes (Figure 2). On the basis of the k_{lim}/K_d values, the magnitude of the switch in specificity for the reductive half-reaction is approximately 82 000-fold in favor of increased specificity for dimethylamine (Figure 4), and this figure compares favorably with the 90 000-fold switch in specificity calculated from the k_{cat}/K_m data (Table 1) in the steady state.

(B) Single Wavelength Studies at 365 nm. As previously noted by Falzon and Davidson (1996a), single wavelength kinetic transients at 365 nm for the wild-type enzyme with trimethylamine are biphasic and both rate constants (k_{obs2} and k_{obs3}) are dependent on substrate concentration. The amplitude of each kinetic phase is also dependent on substrate concentration (Figure 5), which reflects gating of electron transfer at high trimethylamine concentrations. If the rate constants are analyzed separately using eq 2, then the

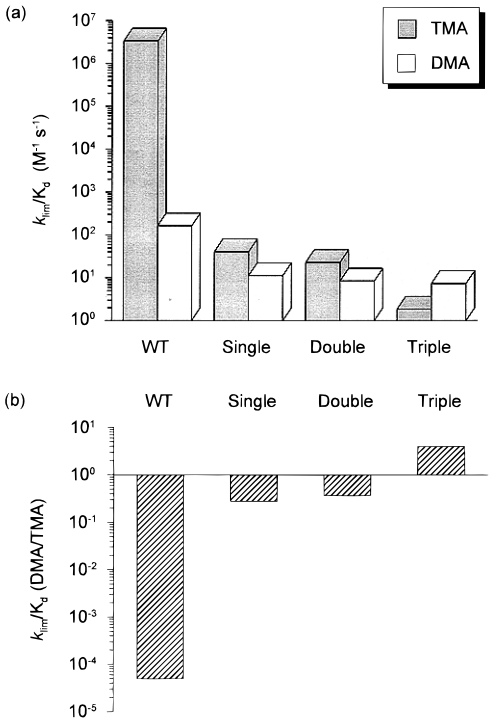

FIGURE 4: Histograms illustrating the effects of mutations on the ratio of the limiting rate constant and dissociation constant for the flavin reduction at pH 7.5 (panel a) and the magnitude of the switch in specificity of the reductive half-reaction (panel b). Key: WT, wild-type enzyme; single, mutant Y60Q; double, mutant Y60Q·S74T; triple, mutant Y60Q·S74T·W105F.

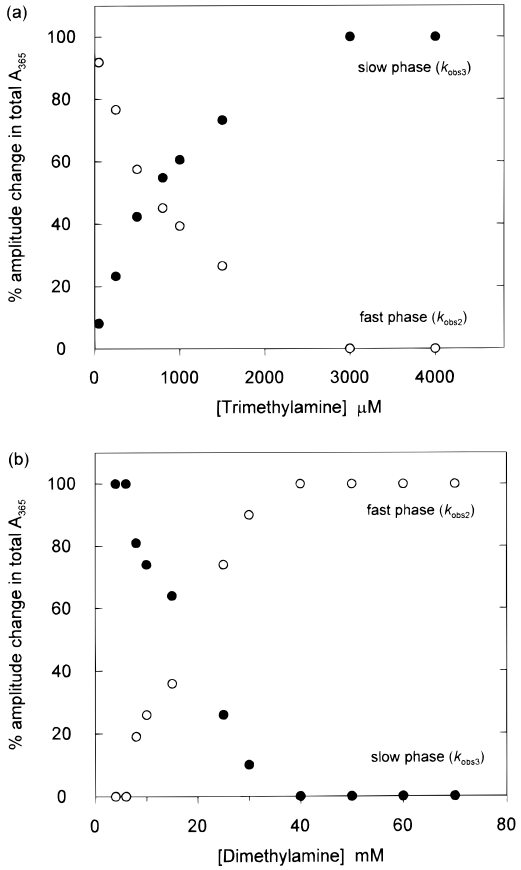

FIGURE 5: Plots illustrating the amplitude of the k_{obs2} and k_{obs3} kinetic phases as a percentage of the total absorbance change observed at 365 nm for the reaction of wild-type TMADH with (a) trimethylamine and (b) dimethylamine.

apparent dissociation constants 188 ± 30 and 350 ± 25 μM and limiting rate constants 89 ± 4 and 6.3 ± 0.1 s^{-1} are calculated for the k_{obs2} and k_{obs3} phases, respectively. These kinetically determined K_d values are similar and may reflect the same processes in the reductive half-reaction, but their true significance remains uncertain (Falzon & Davidson, 1996a).

With dimethylamine as substrate, the wild-type enzyme also displays biphasic 365 nm transients. In this case, the rate constants (k_{obs2} and k_{obs3}) were apparently dependent on substrate concentration over the range used in these studies (Table 3). However, given the limited data for each rate constant over the substrate range used, no attempt was made to calculate values for the apparent dissociation constants for each kinetic phase (Table 3). The amplitudes for the k_{obs2} and k_{obs3} phases change as a function of substrate concentration and are opposite in trend to those seen for the wild-type enzyme/trimethylamine data (Figure 5). The data strongly suggest that gating of electron transfer is not responsible for the biphasic kinetics. Recall also that in the steady state inhibition was not seen with dimethylamine as substrate, also suggesting that gating mechanisms are not operative with this substrate. A comparison was made of the rate constants for the fast phase of the reductive half-reaction (measured at 443 nm) with the two rate constants for the absorbance changes at 365 nm over the same range of substrate concentration (Table 3). From this analysis, it was clear that the dominance of k_{obs2} in the biphasic data at 365 nm is controlled by the rate of flavin reduction (i.e., substrate-dependent changes at 443 nm). At low dimethylamine concentrations (e.g., 4 mM), the low rate of flavin reduction (0.11 s^{-1}) controls subsequent changes at 365 nm, such that the k_{obs3} rate dominates. As the rate of flavin reduction is increased at higher dimethylamine concentrations, faster rates of internal electron transfer are possible, and the k_{obs2} rate begins to dominate. The data clearly indicate that mixing of the 443 and 365 nm kinetic phases occurs when the rate of flavin reduction is compromised, and it explains in the rapid-scanning experiments why spectral deconvolution of the true intermediates was not possible using global analysis/numerical integration methods for wild-type TMADH with dimethylamine.

Table 3: Rate Constants for Absorbance Changes at 365 nm for the Wild-Type Enzyme with Dimethylamine as Substrate[a]

dimethylamine concn (mM)	k_{obs2} (s^{-1})	k_{obs3} (s^{-1})	amplitude: first phase (% total A_{365})	amplitude: second phase (% total A_{365})	k_{obs1} (443 nm) (s^{-1})
4		0.11	0	100	0.11
6		0.17	0	100	0.16
8	nd	0.23	19	81	
10	nd	0.27	26	74	0.51
15	1.29	0.45	36	64	
25	1.38	nd	74	26	1.14
30	1.47	nd	90	10	1.54
40	1.83		100	0	
50	1.74		100	0	3.15
60	1.83		100	0	3.7
70	1.93		100	0	4.17

[a] All reactions were performed at 30 °C and pH 7.5. nd = not determined because amplitude changes were too small for an accurate evaluation of this rate constant.

All the mutant enzymes with dimethylamine and trimethylamine as substrate (with the exception of the Y60Q mutant with trimethylamine) exhibited monophasic absor-

Table 4: Limiting Rate Constants and Apparent Dissociation Constants for the Monophasic Absorbance Changes Occurring at 365 nm for Mutant TMADH Enzymes with Dimethylamine and Trimethylamine as Substrate[a]

	trimethylamine		dimethylamine	
enzyme	k_{lim} (s^{-1})	K_d (mM)	k_{lim} (s^{-1})	K_d (mM)
Y60Q	2.0 ± 0.1	31 ± 6	1.2 ± 0.1	64.5 ± 8
Y60Q·S74T	2.3 ± 0.2	73 ± 10	1.5 ± 0.2	150 ± 29
Y60Q·S74T·W105F	0.2 ± 0.02	140 ± 26	1.1 ± 0.1	176 ± 37

[a] All reactions were performed at 30 °C and pH 7.5.

bance changes at 365 nm that were dependent on substrate concentration (Table 4). In all these cases, kinetic mixing with the phase attributed to flavin reduction (443 nm) is observed. From Table 2, it is apparent that all the limiting rate constants for flavin reduction are equal to or less than 2 s^{-1}: the rate constants for flavin reduction over the same range of substrate concentrations used in the 365 nm experiments are substantially less than 2 s^{-1}. Consequently, the intermediate kinetic phase for electron transfer (365 nm) is not observed (as it was at high dimethylamine concentrations for the wild-type enzyme), and only the slow kinetic phase (equivalent to k_{obs3} for the wild-type−dimethylamine experiments) is seen. As the rate of flavin reduction is increased by raising the substrate concentration, the rate constants for the changes in absorbance at 365 nm are also increased. By fitting to eq 2, limiting rate constants and apparent dissociation constants were calculated (Table 4), and in all cases these were similar to those calculated for the data collected at 443 nm. This latter observation lends support to the idea that the 365 nm transients are closely related to the substrate-dependent rates of flavin reduction. With the Y60Q mutant reduced with trimethylamine, transients at 365 nm were complicated but could be approximated to monophasic changes. Electron transfer between the flavin and 4Fe-4S center in the Y60Q mutant is attenuated since the enzyme is inhibited by high substrate concentrations in the steady state. However, because of the extra complication due to kinetic mixing of the faster 365 nm absorption changes with the 443 nm phase, true biphasic transients (as seen for the wild-type enzyme with trimethylamine) were not seen at 365 nm. For this reason, we approximated the transients to monophasic changes and analyzed the data using eq 2, the result being that the fit to eq 2 was not as good as those observed with the Y60Q mutant with dimethylamine or the double and triple mutant enzymes with either substrate.

DISCUSSION

Our modeling of the active site of DMADH and TMADH suggested that three changed amino acid residues in DMADH (Gln-60, Thr-74, and Phe-105), in conjunction with other residues found unchanged in both enzymes (Trp-264 and Trp-355), were responsible for the binding of dimethylammonium ions and the rejection of trimethylammonium ions. When these residues were introduced into trimethylamine dehydrogenase by conventional site-directed mutagenesis, the specificity of the triple mutant (Y60Q·S74T·W105F) was switched some 90 000-fold in favor of dimethylamine. Surprisingly, however, the major component of this switch was attributed to a selective impairment in the catalytic efficiency of the enzyme with trimethylamine, the steady-state parameters with dimethylamine being affected to only a small extent compared with those of the wild-type enzyme. Stopped-flow analyses of the wild-type and mutant enzymes revealed that the switch in specificity observed in the steady state is attributable to substantially reduced rates of flavin reduction with trimethylamine and poor binding of this substrate in the active site. Removal of just one of the aromatic residues of the substrate-binding aromatic bowl in TMADH, therefore, has a profound effect on substrate presentation in the active site.

In the mutant enzymes, the rates of flavin reduction with trimethylamine are reduced to the extent that flavin reduction is now at least partially rate-limiting during catalysis. The manifestation of these effects is a mixing of the kinetic phases observed in the stopped-flow studies of the mutant enzymes. Unlike for the wild-type enzyme, the transients observed at 365 nm for the mutant enzymes with trimethylamine were monophasic and dependent on substrate concentration. In the wild type, the 365 nm transients are biphasic, and the relative proportion of each phase is tightly controlled by substrate concentration through an attenuation mechanism for electron transfer between flavin and the 4Fe-4S center (Falzon & Davidson, 1996a,b). This phenomenon gives rise to substrate inhibition in the steady state. The double (Y60Q·S74T) and triple (Y60Q·S74T·W105F) mutant enzymes displayed no tendency for substrate inhibition in the steady state with trimethylamine as substrate, whereas the Y60Q mutant displayed some inhibition at high concentrations of substrate (K_i = 2.6 mM). These findings are consistent with the simple monophasic transients observed at 365 nm for the double and triple mutants when reduced with trimethylamine. In these cases, the apparent substrate dependency at 365 nm is a result of kinetic mixing of transients observed at 443 nm (substrate dependent) with those at 365 nm. For the Y60Q mutant, however, the data at 365 nm could at best only be approximated to simple exponential processes, and fits of the observed rate constant to eq 2 were poor. Due to kinetic mixing with the 443 nm phase, a good biphasic transient (as seen for the wild-type enzyme which shows gating of internal electron transfer) at 365 nm was not observed with the Y60Q mutant.

With dimethylamine as substrate, our investigations have shown that in the wild-type enzyme the poor rates of flavin reduction also give rise to kinetic mixing effects during studies of intramolecular electron transfer. However, in this case, the rate of flavin reduction, especially at relatively high dimethylamine concentrations, is sufficiently fast to allow expression of the faster rate for intramolecular electron transfer as well as the slow rate attributable to further reduction of the 4Fe-4S center and formation of the spin-interacting state. The transients at 365 nm are therefore biphasic, the faster rate, as expected, being more dominant at higher dimethylamine concentrations. The biphasic kinetics are not a result of substrate-dependent attenuation of intramolecular electron transfer (as seen for the wild-type enzyme with trimethylamine), since the relative amplitude changes of the two phases show the opposite trend as a function of dimethylamine concentration to those observed with trimethylamine. Also, in the steady state, substrate inhibition was not observed for the wild-type enzyme with dimethylamine. In the mutant enzymes with dimethylamine, the rate of flavin reduction is impaired by a factor of about 20-fold compared with the wild-type enzyme. In these cases, the rates of intramolecular electron transfer are more tightly

controlled by the rate of flavin reduction giving rise to monophasic transients at 365 nm; i.e., expression of the faster 365 nm rate seen for the wild-type enzyme with dimethylamine is prevented. The 365 nm transients for the mutant enzymes are dependent on substrate concentration in much the same way as was observed for the double and triple mutant enzymes with trimethylamine.

In the triple mutant (Y60Q·S74T·W105F) enzyme, which displays the largest (90 000-fold) switch in specificity in favor of dimethylamine, the dissociation and Michaelis constants for dimethylamine are still poor (105 and 7.25 mM, respectively) and comparable to those measured for the wild-type enzyme. In this regard, the engineering experiments did not satisfy fully the predictions made from our modeling work, even though the activity with trimethylamine was severely compromised. In the absence of a structural solution for any of the mutant enzymes, we conjecture that the hydrogen bond formed by the N−H group of dimethylamine and the $O_{\epsilon 1}$ of the glutamine residue in the model of DMADH is not formed in the mutant enzyme−substrate complex. Although the active sites of DMADH and the mutant TMADH are identical at the level of residue identity, we cannot disregard any long-range effects that may perturb the active site structure in the mutant TMADH. These long-range effects may prevent formation of the hydrogen bond from the engineered glutamine to substrate in the mutant TMADH enzymes. Any subtle long-range effects on the active site structure will only be realized following a structural determination of the mutant TMADH enzymes, and these experiments will now become the focus for future work.

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