Deuterium Isotope Effects during Carbon–Hydrogen Bond Cleavage by Trimethylamine Dehydrogenase

IMPLICATIONS FOR MECHANISM AND VIBRIONALLY ASSISTED HYDROGEN TUNNELING IN WILD-TYPE AND MUTANT ENZYMES

Received for publication, February 7, 2001, and in revised form, March 29, 2001
Published, JBC Papers in Press, April 13, 2001, DOI 10.1074/jbc.M101178200

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His-172 and Tyr-169 are components of a triad in the active site of trimethylamine dehydrogenase (TMADH) comprising Asp-267, His-172, and Tyr-169. Stopped-flow kinetic studies with trimethylamine as substrate have indicated that mutation of His-172 to Gln reduces the limiting rate constant for flavin reduction ~10-fold (Basran, J., Sutcliffe, M. J., Hille, R., and Scrutton, N. S. (1999) Biochem. J. 341, 307–314). A kinetic isotope effect (KIE = kH/kD) accompanies flavin reduction by H172Q TMADH, the magnitude of which varies significantly with solution pH. With trimethylamine, flavin reduction by H172Q TMADH is controlled by a single macroscopic ionization (pKs = 6.8 ± 0.1). This ionization is perturbed (pKs = 7.4 ± 0.1) in reactions with perdeuterated trimethylamine and is responsible for the apparent variation in the KIE with solution pH. At pH 9.5, where the functional group controlling flavin reduction is fully ionized, the KIE is independent of temperature in the range 277–297 K, consistent with vibrationally assisted hydrogen tunneling during breakage of the substrate C–H bond. Y169F TMADH is ~4-fold more compromised than H172Q TMADH for hydrogen transfer, which occurs non-classically. Studies with Y169F TMADH suggest partial thermal excitation of substrate prior to hydrogen tunneling by a vibrationally assisted mechanism. Our studies illustrate the varied effects of compromising mutations on tunneling regimes in enzyme molecules.

Quantum tunneling effects in enzymatic hydrogen transfer reactions are being observed in a growing number of enzyme systems (1–13). Some have been modeled using the classical formulations of transition state theory (14), incorporating a tunneling correction that accounts for tunneling below the saddle point of the potential energy surface (15). Recently, however, experimental observations have suggested that tunneling in some enzymes is vibrationally assisted (6, 7, 12, 13, 16, 17), and the potential energy barrier to the reaction should thus be seen as fluctuating rather than static. The fluctuating potential energy surface derives from the thermal vibrations of the protein, which drive the hydrogen tunneling reaction (for recent reviews, see Refs. 18–21). Theoretical treatments of vibrationally assisted tunneling reactions have been presented, with the dynamic component reflecting substrate vibrations, protein vibrations, or both. Antoniou and Schwartz (22) and Borgis and Hynes (23) have provided theoretical descriptions of hydrogen tunneling facilitated by vibrations in the substrate. Bruno and Bialek (24) proposed that hydrogen tunneling is coupled to protein vibrations, but their treatment is specific for hydrogen transfer from the substrate vibrational ground state. Kuznetsov and Ulstrup (25) have provided a more general treatment of enzymatic hydrogen tunneling involving coupling between the tunneling modes and the environment. In the context of vibrationally assisted tunneling mechanisms, major experimental challenges are now presented. These include correlation of (i) protein flexibility with the degree of tunneling (26), (ii) barrier shapes and tunneling regimes with compromised rates of transfer with “slow” substrates and in mutant enzymes (12), and (iii) experimental data with computational studies of the quantum dynamics of hydrogen transfer (27).

In this work, we have extended our studies of hydrogen tunneling to the iron-sulfur flavoenzyme trimethylamine dehydrogenase (TMADH); EC 1.5.99.7, which catalyzes the oxidative demethylation of trimethylamine to dimethylamine and formaldehyde (28): (CH3)3N + H2O → (CH3)2NH + CH2O + 2H+ + 2e−. The reaction is initiated by the cleavage of a C–H bond in one of the substrate methyl groups, and the reducing equivalents are transferred to a 6-S-cysteinyl-FMN in the enzyme active site (29). In stopped-flow studies, a large primary kinetic isotope effect (KIE) accompanies flavin reduction in native TMADH (30). Following bond cleavage, electrons are transferred subsequently from the dihydroflavin in two single-electron transfer events to electron-transferring flavoprotein via the [4Fe-4S] center of TMADH (31–36). The reductive half-reaction of TMADH is resolved into three kinetic phases (37, 38): a fast phase representing two-electron reduction of the flavin, an intermediate phase that reports on intramolecular electron transfer from dihydroflavin to the [4Fe-4S] center to generate flavin semiquinone and reduced iron-sulfur center, and a slow phase that involves formation of a spin-interacting state of the enzyme and product release (32–34, 38). Flavin reduction in native TMADH is fast, prohibiting detailed studies of flavin reduction at elevated temperatures (38). In this study, we present the first analysis of vibrationally assisted hydrogen tunneling in two mutant forms of TMADH that have compromised rates of flavin reduction and are thus accessible to study by stopped-flow methods.

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¶ The abbreviations used are: TMADH, trimethylamine dehydrogenase; KIE, kinetic isotope effect.
**EXPERIMENTAL PROCEDURES**

Trimethyl-$d_{15}$-amine HCl (99.7% deuterium; chemical purity > 99% as determined by high performance liquid chromatography, NMR, and gas chromatography) was from CK Gas Products Ltd. Titration curves for protiated and deuterated trimethylamine HCl were generated using 20 mM solutions (5 ml) of each tertiary amine at 19.5 °C. The pH of the solution was measured using a Mettler Toledo Inlab 410 electrode after sequential addition of sodium hydroxide from a concentrated stock (100 mM). All chemicals were of analytical grade where possible. Wild-type TMADH was purified from *Methylophilus methylotrophus* (sp. W,) as described by Steenkamp and Mallinson (28), but with the modifications of Wilson et al. (39). The isolation, expression, and purification of H172Q and Y169F TMADH have been described previously (30, 40).

Stopped-flow experiments with TMADH were performed using an Applied Photophysics SX.18MV stopped-flow spectrophotometer as described elsewhere (30, 38, 40). For single-wavelength studies, data collected at 443 nm were analyzed using nonlinear least-squares regression analysis on an Acorn RISC PC using Spectrakinetics software (Applied Photophysics). Experiments were performed by mixing TMADH contained in buffer of the desired pH with an equal volume of substrate contained in the same buffer at the desired concentration. The concentration of substrate was always at least 10-fold greater than that of TMADH, thereby ensuring pseudo first-order reaction conditions. Transients at 443 nm were analyzed as monophasic decreases in absorption, consistent with previous studies (38). For each substrate concentration, at least five replica measurements were collected and averaged. The error for individual rates measured by fitting to a single transient was <0.5% in all cases, and the error for the rate fitted to averaged transients was <0.4%. Substrate-reduced TMADH is quite stable to reoxidation by molecular oxygen (half-life of >50 min (39)), negating the need to use anaerobic conditions in the kinetic experiments.

Reaction scheme modeling for flavin reduction by trimethylamine has been described in detail elsewhere (38). Flavin reduction involves the reversible formation of a Michaelis complex, followed by the C–H bond cleavage/flavin reduction step (Equation 1).

$$k_1 \quad E + S \rightarrow ES \quad \rightarrow EP \quad (\text{Eq. 1})$$

The observed rate of flavin reduction ($v_{obs}$) is dependent on substrate concentration, as described by the general hyperbolic expression in Equation 2 (41).

$$v_{obs} = \frac{k_3[S]}{K + [S]} \quad (\text{Eq. 2})$$

where $K$ is a constant and equal to ($k_2 + k_{b2}/K_c$. As discussed elsewhere (38), in reactions of TMADH with trimethylamine, the reaction is modeled such that $k_2 >> k_{b2}$, and $K$ thus approximates to the enzyme-substrate dissociation constant, $K_c$. For multiple-wavelength stopped-flow studies, the reaction was monitored using an Applied Photophysics photodiode array detector and operated using XSCAN software. Analysis of photodiode array data was carried out using PROKIN software (Applied Photophysics). TMADH is stable over the temperature range used in the stopped-flow studies. This is evident since the total absorption change for 6-S-cysteylin-FMN reduction at all temperatures remains constant and is identical to that observed in spectrophotometric titrations of the enzyme with its substrate. Enzyme and substrate were equilibrated for 10 min in the stopped-flow apparatus at the appropriate temperature prior to mixing and the acquisition of stopped-flow data. The optimal time for equilibration was determined empirically. Temperature control was achieved using a thermostatic circulating water bath, and the temperature was monitored directly in the stopped-flow apparatus using a semiconductor sensor (Model LM35CZ, National Semiconductor). In studies of the temperature dependence of bond cleavage, all substrates were used at saturating concentrations (see “Results and Discussion”). Studies of the concentration dependence of bond cleavage at 5 and 35 °C indicated that the value of $K$ in Equation 2 was not substantially perturbed upon changing temperature. These control experiments thus ensured that substrate was saturating at all the temperatures investigated in the temperature dependence studies.

Studies of the pH dependence of flavin reduction were conducted at 5 °C. The buffers used were 100 mM potassium phosphate (pH 6.0–7.5), 100 mM sodium pyrophosphate (pH 8.0–8.5), 100 mM sodium borate (pH 9.0–10.0), and 100 mM glycine/NaCl (pH 10.5 and 11.0). Previous studies have established that increased ionic strength upon addition of substrate (trimethylammonium chloride) over the range employed in this study does not affect the rate of flavin reduction (39). pH profiles for the kinetic parameters $k_3$ and $K$ were constructed, and the data were fitted to Equations 3 and 4, respectively, to obtain the relevant $pK_a$ values.

$$h_3 = \frac{E[H] \times 10^{-pK_a} + E \times 10^{-pK_a}}{10^{-pK_a} + 10^{-pK_a}} \quad (\text{Eq. 3})$$

$$h_3 = \frac{T_{max}}{1 + 10^{pK_a - pH - 10^{pK_a - pH}} + 10^{pK_a - pH - 10^{pK_a - pH}}} \quad (\text{Eq. 4})$$

where $E$ and $H$ are the catalytic activities of the protonated and unprotonated forms of the ionization group, respectively; and $T_{max}$ is the theoretical maximal value of $k_3/K$. The optimal time for equilibration was determined empirically. Temperature control was achieved using a thermostatic circulating water bath, and the temperature was monitored directly in the stopped-flow apparatus using a semiconductor sensor (Model LM35CZ, National Semiconductor). In studies of the temperature dependence of bond cleavage, all substrates were used at saturating concentrations (see “Results and Discussion”). Studies of the concentration dependence of bond cleavage at 5 and 35 °C indicated that the value of $K$ in Equation 2 was not substantially perturbed upon changing temperature. These control experiments thus ensured that substrate was saturating at all the temperatures investigated in the temperature dependence studies.

**RESULTS AND DISCUSSION**

**pH Dependence of Flavin Reduction with Trimethylamine and Perdeuterated Trimethylamine**—Previously, we analyzed the pH dependence of flavin reduction by trimethylamine with H172Q TMADH at 5 °C (30). This study revealed the presence of a single kinetically influential ionization ($pK_a = 6.8 \pm 0.1$) in the enzyme-substrate complex. Similar studies with native TMADH are compromised owing to the very fast flavin reduction rates (>1200 s$^{-1}$ (38)). We have, however, analyzed the pH dependence of the native enzyme using perdeuterated trimethylamine (30) since the primary KIE ($k/H$) upon C–H bond cleavage/flavin reduction allowed us to analyze the full kinetic transient on the stopped-flow time scale. In native TMADH, there are two kinetically influential ionizations in the enzyme-substrate complex, and their origin is of interest from a mechanistic viewpoint. The upper ionization is attributed to the side chain of His-172 (since it is lost in H172Q TMADH (30)); the lower ionization is as yet unassigned, but it most likely represents the deprotonation of the trimethylammonium cation ([CH$_3$]$_3$NH$^+$) to form trimethylamine base. Deprotonation of the substrate cation is consistent with a mechanism of flavin reduction in which the substrate nitrogen lone pair undergoes nucleophilic addition at the flavin C-4a atom (Fig. 2) (42). This mechanism is analogous to that proposed recently for monoamine oxidase A on the basis of structure-activity relationships with para-substituted benzylamine analogs (43). All ionizable groups in the active site of TMADH (His-172 (30), Tyr-169 (40), Tyr-174, and Tyr-60) have been mutated without loss of this ionization, thus supporting assignment to the deprotonation of substrate itself in the enzyme-substrate complex.

In this study, we have performed an analysis of the pH dependence of flavin reduction in H172Q TMADH using perdeuterated substrate (Fig. 1 and Table I). The plot of $k_3$ versus $pH$ retains the features we reported previously for H172Q TMADH in reactions with trimethylamine as substrate (shown also in Fig. 1A), but the macroscopic $pK_a$ describing the kinetically influential ionization in the enzyme-substrate complex is shifted from 6.8 ± 0.1 (trimethylamine) to 7.4 ± 0.1 (perdeuterated trimethylamine). The isotope dependence of this $pK_a$ value is of interest. Although, in the literature, this $pK_a$ value has not been attributed formally to a group in the enzyme-substrate complex, as discussed above, the balance of evidence suggests that it represents the deprotonation of the trimethylammonium cation.

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**Notes:**

1. Basran, J. M. Sutcliffe, and N. S. Scrutton, unpublished data.

2. Previous studies have established that increased ionic strength upon addition of substrate (trimethylammonium chloride) over the range employed in this study does not affect the rate of flavin reduction (39).
substrate molecule itself ((CH₃)₃NH⁺ → (CH₃)₃N) on moving from low to high pH. It is anticipated that perdeuteration of the substrate will affect this ionization (and see below). (i) the shorter C–D bond results in a larger charge density, and thus, it is electron-supplying (i.e., stabilizing the N–H bond) relative to C–H; and (ii) the perdeuterated substrate has a greater reduced mass for the (CD₃)₃N–H stretching vibration and therefore lies lower in the asymmetric potential energy well. Thus, the (CH₃)₃N–H bond dissociates more readily than the (CD₃)₃N–H bond, accounting for the elevated macroscopic pKₐ value seen with perdeuterated substrate in our kinetic studies.

The KIE for C–H bond breakage in H172Q TMADH across the pH range is illustrated in Fig. 1C. Inflated KIE values (>7) are seen in the low pH range. These result from the isotopic dependence, kinetically influential ionization in the enzyme-substrate complex (Fig. 1A). Scheme 1 summarizes the prototropic control of flavin reduction in the enzyme-substrate complex. In Scheme 1, it is assumed that the rate of breakdown of the ES complex to E is slow relative to the dissociation steps, so the dissociation steps remain in thermodynamic equilibrium. Clearly, as a result of the elevated pKₐ value seen with perdeuterated substrate, there is a greater concentration of the ES⁻ (unreactive) complex (i.e., the lower branch of Scheme 1). The effect of this partitioning between ES and ES⁻ forms of the enzyme-substrate complex is that the observed KIE is inflated over the intrinsic value that would be realized if the concentration of the ES species were equivalent (at a given pH value) for both perdeuterated and protiated substrate. Only at pH values of 9.5 and above (where the group identified in the plot of kₐ/K versus pH is fully ionized and where the rate of flavin reduction is maximal) is the intrinsic isotope effect realized, owing to the enzyme being in the ES form for both protiated and perdeuterated substrate. In this regime, the KIE approaches a constant value of ~4.5 (Fig. 1C and Table I).

Fig. 1B illustrates the plot of kₐ/K versus pH for both protiated and perdeuterated substrate. Again, the general features of the plot are retained for both substrates, indicating that two ionizations in the free enzyme and/or free substrate are kinetically influential. The assignments of the ionizations in the kₐ/K plot are unknown. Previously, we hypothesized that the acid limb of the bell-shaped curve might reflect ionization of Tyr-60, which contacts the substrate in the enzyme-substrate complex (38). However, studies with the Y60F mutant now indicate this is not the case since the bell-shaped dependence of the kₐ/K plot was still observed in this mutant.² We also conjectured (again without formal demonstration) that the alkaline limb might represent the deprotonation of trimethylammonium cation when not complexed to the enzyme. This suggestion was made since it was initially thought that the substrate cation was the catalytically active species, whereas based on the isotope evidence presented above, computational studies (42), and the mechanism of inactivation of TMADH with phenylhydrazine (42, 44) and by analogy with the mechanism proposed for monoamine oxidase A (43), it now seems likely that unprotonated substrate is the active species. The pKₐ value for deprotonation of trimethylammonium cation is ~9.8, which is close to the values on both the acid and alkaline limbs of the curve generated from fitting to the plot of kₐ/K versus pH. It thus seems appropriate to reevaluate the assignments of the two ionizations in the plot of kₐ/K versus pH, especially in the light of the proposed mechanism in which a complex between trimethylamine base and enzyme is the catalytically active species (Fig. 2). In such a scenario, it clearly makes more sense for the enzyme to have evolved a higher affinity for trimethylamine base rather than its protonated cation. Improved binding (in terms of pKₐ) is observed on moving from the acid to alkaline region of the lower (acid) limb of the kₐ/K versus pH plot, and this ionization therefore most likely represents deprotonation of the substrate cation. The upper, alkaline limb remains unassigned, but as discussed above, extensive mutagenesis studies have failed to identify a chemical group in the enzyme active site responsible for this ionization; a possible candidate is the N₃H position of the flavin isooxalazine ring (pKₐ ~ 10). With H172Q TMADH, the enzyme-substrate dissociation constants measured across the pH range are modestly elevated with perdeuterated trimethyl-
amino relative to protonated substrate (Table I). Again, as discussed above, these small differences are likely related to the different chemical properties of the (CD₃)₃N–H and (CH₃)₃N–H bonds, which will affect the pKₐ for the ionization of the cationic form of the substrate (pKₐ for (CH₃)₃N–H = 9.8 and pKₐ for (CD₃)₃N–H = 10.1). This will reduce the concentration of the unprotonated form of perdeuterated substrate compared with unprotonated protonated substrate at a given pH value, giving rise to an apparent increase in the enzyme-substrate dissociation constant (since substrate concentration is measured as the sum of the cationic and free base forms). The elevated pKₐ for deprotonation of perdeuterated substrate is consistent with the observed shift in the acid limb of the k/K versus pH plot relative to the curve for protonated substrate (Fig. 1B). This strengthens the assertion that the acid limb is attributable to the deprotonation of substrate, which is consistent with the mechanism shown in Fig. 2.

Vibrationally Assisted Hydrogen Tunneling in TMADH—Studies of the temperature dependence of the KIE can be used to indicate whether hydrogen transfer occurs classically or by quantum tunneling (6). The temperature dependence of the rates of C–H and C–D bond cleavage is described by the unimolecular rate (Equation 5),

$$k = \frac{k_{\text{H}}}{h} T_{\text{a}} e^{-\Delta H^*/RT} = \frac{k_{\text{D}}}{h} T_{\text{a}} e^{-\Delta H^*/RT} e^{\Delta A^*/RT}$$

(Eq. 5)

where kₜₜ and h are the Boltzmann and Planck constants, respectively. Temperature-dependent rate data can be plotted conveniently using the following form of the Eyring equation (Equation 6).

$$\ln(k/hT) = \ln(k_T/h) + \Delta S^*/R - \Delta H^*/RT$$

(Eq. 6)

The enthalpy of activation (ΔH*) is calculated from the slope of the plot; ΔS* is calculated by extrapolation to the ordinate axis, and ΔG* is then calculated directly from Equation 5. The use of Equation 6 in plotting the temperature dependence of a unimolecular reaction is preferred over the use of the classical Arrhenius plot. This arises because the Arrhenius equation is curved (although it appears linear in the accessible temperature range) and asymptotically approaches infinity at high temperatures. Use of the Arrhenius plot has led to the development of criteria to indicate tunneling based on the values for ΔΔEₙ (the difference in the Arrhenius activation energies for protium versus deuterium transfer) and the Aₜₜ/AₜD ratio (calculated from the intercepts of the Arrhenius plot for protium and deuterium substrates) (4). The corresponding parameters calculated from the slopes and intercepts of plots using Equation 6 are ΔΔH = ΔHₜₜ/ΔHₜD and Aₜₜ/AₜD (the primes are used to distinguish this ratio from the Aₜₜ/AₜD ratio calculated from the Arrhenius plot). Aₜₜ and AₜD are the intercept values, and the value of the ratio of Aₜₜ to AₜD is a measure of the extent of tunneling. For semiclassical reactions, Aₜₜ/AₜD is unity; for reactions that occur by vibrationally assisted tunneling from the substrate vibrational ground state, the value of the Aₜₜ/AₜD ratio is equal to the value of the intrinsic primary KIE (6).

The temperature dependence of flavin reduction in H172Q
TMADH with protiated and perdeuterated trimethylamine is illustrated in Fig. 3, and parameters are given in Table II. Reactions were performed using 10 mM substrate, thus ensuring saturation of the enzyme ($K^H = 0.19 \text{ mM}$ and $K^D = 0.44 \text{ mM}$) (Table I), and at pH 9.5 to facilitate complete formation of the catalytic ES (and not $ESH^-$) complex. A striking feature of the plot shown in Fig. 3 is the temperature dependence of the KIE ($\Delta H^H = 0.5 \pm 5.2 \text{ kJ mol}^{-1}$) over the temperature range investigated. The value of the $A^{H:A^D}$ ratio (7.8 ± 1.0) is similar to the KIE (4.6 ± 0.4), which is suggestive of hydrogen and deuterium tunneling from the substrate ground state, but reaction rates are clearly still dependent on temperature ($\Delta H^T \approx 41 \text{ kJ mol}^{-1}$), consistent with a need to assist the tunneling reaction through vibrational excitation of the protein. The behavior is similar to observations we have published previously in our studies of C–H and C–D bond cleavage in tryptophan tryptophylquinone (TTQ)-dependent amine dehydrogenases (6, 12) and in a heterotetrameric sarcosine oxidase (7) and also to that seen by others in thermophilic alcohol dehydrogenase (16) and acyl-CoA desaturase (13); these findings have also been rationalized in terms of vibrationally assisted tunneling of hydrogen and deuterium. The transfer of the hydrogen and deuterium nuclei (from the substrate vibrational ground state) by a vibrationally assisted tunneling mechanism in a compromised mutant (H172Q) of TMADH has clear implications for hydrogen and deuterium transfer in native enzyme. Although the temperature dependence of the KIE cannot be measured in native TMADH at pH 9.5 owing to the very fast limiting rates (>$1200 \text{ s}^{-1}$ (38)) of hydrogen transfer, we have been able to perform a study of the temperature dependence of deuterium transfer (Fig. 3B). Analysis of these data indicates that $\Delta H^D$ (45.7 ± 0.9 kJ mol$^{-1}$) in native TMADH is similar to $\Delta H^D$ (41.2 ± 2.6 kJ mol$^{-1}$) and $\Delta H^D$ (41.7 ± 2.6 kJ mol$^{-1}$) for H172Q TMADH, indicating a similar energetic requirement to distort the protein geometry into one that is compatible with hydrogen and deuterium tunneling. The compromised reaction rates in the H172Q mutant relative to native enzyme are therefore likely attributable to a widening of the potential energy barrier for C–H/C–D bond cleavage in the optimally configured enzyme-substrate complex. These findings are qualitatively similar to our recent studies with TTQ-dependent aromatic amine dehydrogenase with a fast (tryptamine) and slow (dopamine) substrate (12); in both cases, hydrogen transfer was inferred to be from the substrate vibrational ground state, and the compromised rates observed with dopamine were attributed to a broadening of the potential energy barrier in the optimal (thermally activated) geometry of the enzyme-substrate complex.

We have also investigated the temperature dependence of the KIE in Y169F TMADH. This mutant is 4-fold more compromised in its limiting rate of flavin reduction relative to the H172Q mutant, and analysis of the temperature dependence of C–H and C–D bond cleavage was performed to probe the effect of further reduction in flavin reduction rate on the hydrogen and deuterium tunneling mechanism. Our previous studies with Y169F TMADH indicated that both ionizations seen with native TMADH (perdeuterated substrate) in the $k_D$ versus pH plot are retained in the mutant TMADH (40). The more acidic ionization has a similar $pK_a$ value in both enzymes (~6.5), whereas the more alkaline ionization is perturbed to a higher pH value in Y169F TMADH ($pK_a = 9.5$ (40)) relative to native TMADH ($pK_a = 8.4$ with perdeuterated substrate (30)). This perturbation has been attributed to the effects of removing a hydrogen bond interaction from the phenolic hydroxy group of Tyr-169 to the imidazole side chain of His-172 in Y169F TMADH, resulting in displacement of the $pK_a$ for the imidazole side chain to a higher pH value (40). A consequence of the perturbed ionization of His-172 in Y169F TMADH is the need to perform temperature dependence studies of C–H and C–D bond cleavage at a higher pH value (pH 11) than that used for corresponding studies with H172Q TMADH (pH 9.5) to ensure full formation of the catalytically active enzyme species (ES). Studies with Y169F TMADH were therefore performed at pH 11 with 10 mM substrate ($K_d = 125 \mu\text{M}$ (40)). Corresponding studies with native TMADH (perdeuterated substrate) were also performed to enable comparison with data collected at pH 9.5 for native and H172Q TMADH.

Our analysis of Y169F TMADH indicates that, unlike with H172Q TMADH, the KIE is not independent of temperature ($\Delta H^T = 3.02 ± 2.5 \text{ kJ mol}^{-1}$) (Fig. 4). Also, the $A^{H:A^D}$ ratio (2.5 ± 0.2) calculated from the intercept of the temperature dependence plot is elevated over that expected for semiclassical transfer ($A^{H:A^D} = \text{unity}$). The enthalpic contributions are similar to those seen with H172Q TMADH at pH 9.5. The data are consistent with a quantum tunneling mechanism, but dif-

![Image](65x302 to 282x730)
The enthalpic contribution to deuterium transfer (and by inference, hydrogen transfer) in native TMADH (which is assumed to transfer via a vibrationally assisted mechanism from the substrate ground state by inference from the H172Q TMADH data) is similar at pH 11 (48.2 ± 0.7 kJ mol⁻¹) and pH 9.5 (45.7 ± 0.9 kJ mol⁻¹). It is worth noting that at any single pH value, the enthalpic contribution for native TMADH is larger than that for the mutant enzymes by ~3–4 kJ mol⁻¹ (Table II). The barrier in native TMADH is therefore more rigid than the corresponding barriers in the mutant enzymes. This may reflect the removal of the side chain hydrogen bond between Tyr-169 and His-172 in each mutant enzyme, making the active site less restrained and thus more readily deformed.

Our analysis of H172Q and Y169F TMADH establishes a link between protein structure and tunneling characteristics for a vibrationally assisted tunneling mechanism. Kliman and co-workers (45) have also established a link between the extent of hydrogen tunneling and mutations in the active site of horse liver alcohol dehydrogenase, and this is similarly likely to reflect increased barrier widths (as revealed through crystallographic analysis of mutant enzymes (45, 46)) and altered dynamics in the active site of the mutant enzymes. Our own studies reported herein and our analysis of the reduction of aromatic amine dehydrogenase with slow substrates (12) suggest that enzymes can tolerate increases in barrier size when catalyzing hydrogen and deuterium transfer by quantum tunneling. Transfer from the substrate vibrational ground state can occur for small increases in barrier width (and therefore relatively small reductions in reaction rate). For very slow substrates or highly compromised mutants, the barrier may be too wide for transfer from the substrate vibrational ground state level (particularly for deuterium), and partial barrier ascent may be required to narrow the transfer distance. Additionally, as seen in reactions of aromatic amine dehydrogenase with benzylamine (a very slow substrate), the enthalpic contribution for barrier compression may also be raised, which in turn will also affect the rate of transfer (12). Our studies therefore illustrate the varied effects of using slow substrates or engineering compromising mutations into the active site of those enzymes that catalyze hydrogen transfer by vibrationally assisted mechanisms.

**Concluding Remarks**—Deuterium isotope studies with H172Q TMADH suggest that oxidation of substrate proceeds from an enzyme-substrate complex in which trimethylamine base is bound in the active site of the enzyme. The optimal rate of flavin reduction thus requires enzyme to preferentially bind trimethylamine, and not the protonated cation. Kinetic and computational data are consistent with a mechanism involving nucleophilic attack of the substrate nitrogen lone pair at the flavin C-4a atom and C–H bond cleavage initiated by the flavin N-5 atom as proposed for monoamine oxidase A. Temperature dependence studies of the rate of C–H bond cleavage are consistent with a vibrationally assisted tunneling mechanism from the substrate ground state in both H172Q and native TMADH, *i.e.* the C–H and C–D bond breaking processes occur entirely by tunneling effects. Compromised rates of C–H bond cleavage in Y169F TMADH are consistent with a need to thermally acti-

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**Table II**

*Calculated parameters from studies of the temperature dependence of C–H and C–D bond cleavage in native and mutant TMADH enzymes*  
Values were determined by fitting Equation 6 to data shown in Figs. 3 and 4. Errors were determined from curve fitting.

| Enzyme                  | $A^\text{H}$ | $A^\text{D}$ | $A^{\text{H},\text{A}^\text{D}}$ | $\Delta H^\text{H}$ | $\Delta H^\text{D}$ | $\Delta H^\text{D} - \Delta H^\text{H}$ |
|-------------------------|--------------|--------------|-------------------------------|---------------------|---------------------|----------------------------------------|
| Native, pH 9.5           |              |              |                               |                     |                     |                                        |
| H172Q, pH 9.5            | 4.25 × 10⁵ ± 2.66 × 10⁶ | 5.47 × 10⁵ ± 3.90 × 10⁵ | 7.8 ± 1.0                   | 41.2 ± 2.6          | 41.7 ± 2.6          | 0.5 ± 5.2                              |
| Native, pH 11.0          | 5.45 × 10⁵ ± 8.40 × 10⁵ |              |                               |                     |                     |                                        |
| Y169F, pH 11.0           | 1.06 × 10⁷ ± 2.45 × 10⁵ | 4.30 × 10⁵ ± 1.83 × 10⁵ | 2.5 ± 0.2                   | 42.1 ± 0.9          | 45.1 ± 1.6          | 3.0 ± 2.5                              |

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**Fig. 4. Temperature dependence and KIE data for Y169F and native TMADH at pH 11.0.**  
A, temperature dependence plots for Y169F TMADH with trimethylamine (○) and perdeuterated trimethylamine (●). Inset, plot of ln(KIE) versus 1/T. Substrate concentration was 10 mM. B, temperature dependence plot for native TMADH with perdeuterated trimethylamine. Parameters evaluated by fitting to Equation 6 are given in Table II. Substrate concentration was 5 mM.
vate the substrate to enable hydrogen tunneling from an excited vibrational state.

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