Importance of Barrier Shape in Enzyme-catalyzed Reactions

VIBRATIONALLY ASSISTED HYDROGEN TUNNELING IN TRYPTOPHAN TRYPTOPHYLQUINONE-DEPENDENT AMINE DEHYDROGENASES

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C–H bond breakage by tryptophan tryptophylquinone (TTQ)-dependent methyamine dehydrogenase (MADH) occurs by vibrationally assisted tunneling (Basran, J., Sutcliffe, M. J., and Scrutton, N. S. (1999) Biochemistry 38, 3218–3222). We show here a similar mechanism in TTQ-dependent aromatic amine dehydrogenase (AADH). The rate of TTQ reduction by dopamine in AADH has a large, temperature independent kinetic isotope effect (KIE = 12.9 ± 0.2), which is highly suggestive of vibrationally assisted tunneling. H-transfer is compromised with benzylamine as substrate and the KIE is deflated (4.8 ± 0.2). The KIE is temperature-independent, but reaction rates are strongly dependent on temperature. With tryptamine as substrate reaction rates can be determined only at low temperature as C–H bond cleavage is rapid, and an exceptionally large KIE (54.7 ± 1.0) is observed. Studies with deuterated tryptamine suggest vibrationally assisted tunneling is the mechanism of deuterium and, by inference, hydrogen transfer. Bond cleavage by MADH using a slow substrate (ethanolamine) occurs with an inflated KIE (14.7 ± 0.2 at 25 °C). The KIE is temperature-dependent, consistent with differential tunneling of proton and deuterium. Our observations illustrate the different modes of H-transfer in MADH and AADH with fast and slow substrates and highlight the importance of barrier shape in determining reaction rate.

Aromatic amine dehydrogenase (AADH)1 and methyamine dehydrogenase (MADH) catalyze the oxidative deamination of primary amines to their corresponding aldehydes and ammonia.

\[ \text{RCH}_2\text{NH}_2 + \text{H}_2\text{O} \rightarrow \text{RCH}_2\text{O} + \text{NH}_3 + 2\text{H}^+ + 2\text{e}^- \]

**REACTION 1**

The physiological electron acceptors of MADH are amicyanin (for the Paracoccus denitrificans enzyme; Refs. 1–3) or a c-type cytochrome (for the Methylphilus methylotrophus enzyme; Ref. 4). AADH transfers electrons (derived from the deamination of aromatic amines) to the copper protein azurin (5). In a reductive half-reaction, both enzymes reduce the prosthetic group tryptophan tryptophylquinone (TTQ) through the initial formation of a carbinolamine intermediate (Fig. 1; Refs. 5 and 6); loss of water from this carbinolamine produces an iminoquinone. The iminoquinone decays by breakage of the substrate C–H bond. An active site base that abstracts a proton from the substrate initiates bond cleavage (7). This step in the reductive half-reaction is rate-limiting, as demonstrated by the unusually large kinetic isotope effect (KIE) observed with methyamine (MADH; Refs. 8–10) and dopamine (AADH; Ref. 11).

Previously we demonstrated that cleavage of the substrate C–H bond of *M. methylotrophus* (sp. W3A1) MADH is associated with a large KIE (16.8 ± 0.5) and that the KIE is independent of temperature (8). Through detailed temperature dependence studies, we were able to suggest that C–H bond breakage occurs by extreme quantum tunneling (8). Quantum tunneling effects in enzymatic hydrogen transfer have been observed in only a small number of enzyme systems (8, 12–21). Most have been modeled using the classical formulations of transition state theory, incorporating a tunneling correction factor to account for tunneling below the saddle-point of the potential energy surface. In our studies of H-tunneling in MADH, we demonstrated that reaction rates were dependent on temperature, thus providing experimental evidence supporting a role for thermally induced, vibrational motion of the protein scaffold in driving enzymatic H-transfer (8). Recently, similar observations have been made with a thermophilic alcohol dehydrogenase (22) and heterotetrameric sarcosine oxidase (17). These observations point to the potential general importance of protein dynamics in driving H-tunneling reactions in enzymes (for recent reviews, see Refs. 23–26) and also to the potential role of low frequency vibrations in facilitating catalysis via the “over-the-barrier” route (27, 28).

In this paper we demonstrate that reduction of the TTQ cofactor by dopamine in AADH also occurs by a vibrationally assisted tunneling mechanism, thus establishing close similarities in the mode of bond cleavage with MADH and its physiological substrate, methyamine (8). We also show that vibrationally assisted tunneling is the likely mechanism of bond cleavage for the reaction of AADH with the slow substrate, benzylamine. Compromised reaction rates with this substrate are suggested to be a consequence of (i) increased width of the potential energy barrier and (ii) a larger enthalpy of activation for deformation of the protein structure to produce geometries compatible with H-tunneling. By contrast, we also show that reactions of MADH with a slow substrate (ethanolamine) re-
quire partial aspect of the potential energy surface to facilitate the tunneling reaction, with D-tunneling occurring higher up the potential energy barrier than H-tunneling. Partial aspect of the barrier is required to reduce the tunneling distance and thereby optimize the probability of transfer. These studies highlight the different strategies used by enzymes to facilitate H-transfer by quantum mechanical tunneling.

**Experimental Procedures**

*Enzymes and Substrates—* MADH was purified from *M. methylotrophus* (sp. W3A1) as described previously (29). Following reoxidation of the purified enzyme with 100-fold excess ferricyanide, the enzyme was exchanged into 10 mM potassium phosphate buffer, pH 7.5, by gel exclusion chromatography. Enzyme concentration was determined using an extinction coefficient of 25,200 M⁻¹ cm⁻¹ at 440 nm for the oxidized enzyme (29). AADH was purified from *Alcaligenes faecalis* IFO 14479 as described (30). Following enzyme purification, AADH was exchanged into 10 mM BisTris-propane buffer, pH 7.5, by gel exclusion chromatography. Enzyme concentration was determined using an extinction coefficient of 27,200 M⁻¹ cm⁻¹ at 440 nm for the oxidized enzyme (30). Deuterated dopamine HCl (HO₂C₆H₄CH₂CD₂NH₂HCl, 94.4%), deuterated benzylamine HCl (C₆H₅CD₂NH₂HCl, 99.6%), and deuterated tryptamine HCl (triptamine-β-δ-d₅-HCl, 98%) were from CDN Isotopes. Deuterated ethanolamine (ethanol-1,1,2,2-d₄-amine, 98%) was from CK Gas Products Ltd. The chemical purity of the deuterated tryptamine, ethanolamine, benzylamine, and dopamine was determined to be >99% by either high performance liquid chromatography, NMR, or gas chromatography, or a combination of these methods. The suppliers of each of the deuterated compounds performed the analysis of chemical purity.

*Stopped-flow Kinetic Studies—* Stopped-flow experiments were performed using an Applied Photophysics SX.185MV stopped-flow spectrophotometer. For single wavelength studies, data collected at 440 nm (MADH) and 456 nm (AADH) were analyzed using a least squares regression analysis on an Acorn RISC PC using Spectrakinetics software (Applied Photophysics). Experiments were performed by mixing MADH or AADH contained in the desired buffer, 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 enzyme, thereby ensuring pseudo-first order reaction conditions. For each substrate concentration, at least five replicates measurements were collected and averaged. The error for individual rates measured by fitting to a single transient was, in all cases, less than 0.5% of the determined value. Shot-by-shot variability in the determined rate was <5%, and the error for the rate fitted to averaged transients was <0.4% of the determined value. As reported previously for MADH (8) and AADH (5), the absorbance changes accompanying enzyme reduction were monophasic, with a single rate constant obtained from fits of the data to Equation 1.

\[
A = Ce^{-kt} + b \quad \text{(Eq. 1)}
\]

C is a constant related to the initial absorbance, and b is an offset value to account for a nonzero baseline. Transients obtained for reactions of AADH with tryptamine were biphasic and analyzed using a double exponential expression. The fast phase of these transients (>90% of the total amplitude change) exhibited a very large KIE (54.7), and the slow phase is not resolved in reactions of AADH with deuterated tryptamine. The small amplitude of the slow phase and uncertainty, but it may represent hydrolysis of the imine intermediate (intermediate 5; Fig. 1). The small amplitude of the slow phase and studies using stopped-flow photodiode array spectroscopy clearly indicate that the slow phase is not associated with TTQ reduction. The slow phase is not observed in reactions of AADH with deuterated tryptamine. The absorbance changes accompanying enzyme reduction were monophasic, with a single rate constant obtained from fits of the data to Equation 1, but the very early phase (0–30 ms) of the transient was omitted to avoid contamination of the spectral change from any contribution from protiated substrate (the deuterated dopamine was enriched to a minimal level of 94%). Due to the large KIE with dopamine, analysis of the transient from around 30 ms onward using Equation 1 ensured that any spectral change attributable to protiated data was in fact secondary analysis, with remaining virtual, all of the kinetic transient for the deuterated substrate. Although this precaution was taken in data fitting, fits to the entire transient to a single exponential function were excellent (with no obvious deviation in the early time domain) and produced rates identical to those fits performed from 30 ms onwards. These observations suggest that the level of enrichment of the deuterated dopamine was greater than the minimal value (94%) quoted by the supplier. For all substrates of MADH and AADH, the observed rate constants were found to exhibit dependence on substrate concentration and the reductive half-reactions of AADH and MADH were modeled using the following general scheme.

\[
A + B \xrightarrow{C} D
\]

\[
k_1 \quad k_2
\]

\[
k_3
\]

**Reaction 2**

Data for MADH (ethanolamine as substrate) were fitted using the simplified equation described by Hiromi (31) as described previously for MADH with methylvamine as substrate (8, 9).

\[
k_{obs} = 0.5(k_1[S] + h_2 + k_3 - (k_4[S] + k_5 + k_6)^2 - 4 k_3 k_4[S]^2) \quad \text{(Eq. 2)}
\]

Data for AADH were fitted to the simpler equation (Equation 3; Ref. 32), also as described previously for this enzyme (3).

\[
k_{obs} = \frac{k_1[S]}{K + [S]} \quad \text{(Eq. 3)}
\]

Here, K is a constant and equal to \((k_5 + k_6)/h_5\). For multiple wavelength stopped-flow studies, the reaction was monitored using an Applied Photophysics photodiode array detector and operated using XSCAN software. For data analysis of photodiode array, we used PROKIN software (Applied Photophysics). Both AADH and MADH are stable over the temperature range used in the stopped-flow studies. This is evident since the total absorption change for TTQ reduction at all temperatures remains constant and is identical to that observed in spectrophotometric titrations of the enzyme with its substrate. Enzymes were equilibrated for 10 min in the stopped-flow apparatus at the appropriate temperature prior to 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. Studies of the concentration dependence of bond cleavage at 5 °C and 35 °C indicated that the enzyme-substrate dissociation constant (for MADH) and the value of K in Equation 3 (for AADH) were not substantially perturbed on changing temperature. These control experiments thus ensured that substrate was saturating at all the temperatures investigated in the temperature dependence studies with both MADH and AADH.

**Results and Discussion**

*Vibrationally Assisted H-tunneling in AADH—* AADH has a broad specificity for primary amine substrates. Davidson and co-workers (5) have investigated the substrate preference of AADH in steady-state reactions and demonstrated that aromatic substrates are generally preferred over simple aliphatic primary amines. Moreover, a large KIE has been demonstrated with the aromatic substrate dopamine (11). In this paper, we have extended the kinetic analysis with dopamine to include studies of the effects of temperature on the KIE. We have also probed the effects of temperature on the KIEs observed with conventional and deuterated forms of benzylamine and tryptamine. The dependence of [dopamine] on the rate of TTQ reduction in AADH is illustrated in Fig. 2 for both deuterium- and protium-labeled substrate. The data are similar to those reported previously with one exception; the enzyme-substrate dissociation constant with dopamine in the present study is much smaller (17.1 ± 0.8 μM) than that reported previously (132 μM; Ref. 5). The smaller dissociation constant for the Michaelis complex results from the use of buffers of different ionic strengths. Univalent cations are known to elicit spectral changes in AADH, consistent with the reported virtual activation-binding site in AADH (33). In the present work, the [dopamine] dependence of TTQ reduction was performed at low ionic strength (10 mM BisTris propane buffer, pH 7.5), whereas previous studies were conducted at high ionic strength (0.25 M potassium phosphate buffer, pH 7.5; Ref. 5). The ionic strength...
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**Fig. 1. Reaction mechanism for the reductive half-reaction of MADH.** Steps enclosed in the hatched box represent binding steps: rate constants $k_1$ (forward reaction) and $k_2$ (reverse reaction). A similar scheme has been proposed for the reaction of AADH with aromatic primary amines.

[Diagram of the reaction mechanism]

effects are limited to binding since the limiting rate of TTQ reduction is similar in both the low and high ionic strength regimes. The dependence of the TTQ reduction rate on [deuterated dopamine] is also shown in Fig. 2. The term $(k_2 + k_3)/k_1$ is larger with dopamine ($-17 \mu M$) than with deuterated dopamine ($-12 \mu M$). Similar effects were seen with other substrates of AADH (Figs. 3 and 4). The larger $k_3^H$ than $k_3^D$ might account for this observation with AADH (Equation 3). However, there might be an additional contribution arising from the known differences in the chemical nature of the C–H versus the C–D bond (34). This is necessary to explain the difference in $k_3/k_1$ (i.e., an isotope effect on binding) for MADH obtained from fitting to Equation 2 (see Fig. 6 and previous work with methylvamine (Ref. 8)). For example, (i) the smaller effective bond length of C–D versus C–H (the latter has a higher zero point energy and therefore lies higher in the asymmetric potential energy well) results in a smaller effective size of C–D relative to C–H; (ii) the shorter C–D bond results in a larger charge density, and is therefore electron supplying relative to C–H; and (iii) C–H has a higher dipole moment than C–D. The expectation, therefore, is that the combined effects of multiple isotopic substitution will perturb the dissociation of the enzyme-substrate complex. These effects will likely be pronounced in those cases where the isotopically substituted group is the main determinant in formation of the enzyme-substrate complex (as is the case with methylvamine).

Fig. 2 reveals a large kinetic isotope effect for dopamine (12.9 ± 0.2) on TTQ reduction, indicating that, as with MADH, C–H bond breakage is concerted with cofactor reduction2 (Fig. 1). An indication as to whether H-transfer occurs classically or by quantum tunneling can be gained by investigating the temperature dependence of the rates of C–H and C–D bond cleavage using the unimolecular rate Equation 4 (8).

$$k = \frac{\hbar}{h} \frac{1}{T_e} e^{-\Delta G^\ddagger_{RT}} = \frac{\hbar}{h} \frac{k_B}{T_e} e^{-\Delta H^\ddagger_{RT}}$$

(Eq. 4)

$h$ and $\hbar$ are the Boltzmann and Planck constants, respectively.

Temperature-dependent rate data can be plotted conveniently using the following form of the Eyring equation.

$$\ln(k/\alpha) = \ln(\hbar/\alpha) + \Delta S^\ddagger/\alpha - \Delta H^\ddagger/\alpha T$$

(Eq. 5)

The enthalpy of activation $\Delta H^\ddagger$ is calculated from the slope of the plot, $\Delta S^\ddagger$ is calculated by extrapolation to the ordinate axis, and $\Delta G^\ddagger$ is then calculated directly from Equation 4. As discussed previously (8), the use of Equation 5 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 in fact curved (although it appears linear in the accessible temperature range) and asymptotically approaches infinity at high temperatures. A consequence of using Equation 5 is the need to define explicitly the values obtained from such plots. Use of the Arrhenius plot has led to the development of criteria to indicate tunneling based on the values for $\Delta \Delta E^\ddagger$ and the $A^H/A^D$ ratio (calculated from the intercepts of the Arrhenius plot for proton and deuterium substrates). The corresponding parameters calculated from the slopes and intercepts of plots using Equation 5 are $\Delta \Delta H^\ddagger$ and $A^H/A^D$. The use of a different slope ($A^H/A^D$) compared to the slope of the Arrhenius plot (the prime is used to distinguish this ratio from the $A^H/A^D$ ratio calculated from the Arrhenius plot).

Analysis of the temperature dependence of $k_3$, using Equation 5 is illustrated in Fig. 2. The data indicate that the KIE is independent of temperature and that the difference in the enthalpy of activation for proton versus deuterium transfer ($\Delta \Delta H^\ddagger = \Delta H^D - \Delta H^H = 0.7 \pm 1.5$ kJ mol$^{-1}$) is essentially

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2 Although secondary KIEs can make significant contributions to observed KIEs, and therefore affect their interpretation, they should not compromise the analysis in this case. A predicted upper value for the secondary KIE is 1.15 for reactions involving a change in hybridization ($sp^3$ to $sp^2$) (43), which represents ~8% of the observed KIE.
neling is the mechanism of transfer. Moreover, the data are inconsistent with rigid barrier models of H-tunneling since the vibrational assistance (as indicated by the nature of the temperature dependence plots) requires a fluctuating potential energy barrier for the transfer event. Vibrational assistance has been discussed for many years with regard to classical over-the-barrier reactions (35), but only relatively recently has this been applied to enzymatic quantum tunneling reactions. Temperature-independent KIEs with large enthalpies of activation can be interpreted in a number of ways. The dynamic component may reflect substrate vibrations, protein vibrations, or a combination of the two. Both Antoniou and Schwartz (36) and Borgis and Hynes (37) have provided theoretical descriptions of H-tunneling facilitated by vibrations in the substrate. Alternatively, Bruno and Bialek (38) have proposed that H-tunneling is facilitated by vibrations in the protein, and they have derived a relationship between the intrinsic KIE for C–H bond cleavage and temperature for tunneling reactions occurring from the vibrational ground state of the substrate. Importantly, in this treatment the KIE can adopt values below the “semiclassical” limit of 7 (at 25 °C); values in this limit and in systems consistent with tunnelling have recently been obtained experimentally for some enzymes (17, 22). A more general treatment of enzymatic H-tunneling involving coupling between the tunneling modes and the environment and a fluctuating barrier has been described recently by Kuznetsov and Ulstrup (39). Although we acknowledge that the degree and nature of H-tunneling in enzymatic systems is model-dependent, we feel our data are most consistent with the model proposed by Bruno and Bialek. In particular, the lack of an isotope effect on the activation enthalpy for H- and D-transfer suggests to us that normal vibrational modes of the substrate alone are not the major component of the vibrational assistance observed with AADH. We envisage this vibrational assistance comes predominantly from vibrational modes in the enzyme.

Reaction of AADH with Tryptamine and Benzylamine—The very fast rates of C–H bond cleavage catalyzed by AADH with tryptamine as substrate prevented detailed studies of the dependence of the reaction rate on temperature. Studies of the dependence of the rate of TTQ reduction on [tryptamine] at 4 °C revealed a highly inflated KIE (54.7 ± 1.0) for bond cleavage (Fig. 3, panel A). This highly inflated KIE is not the result of inhibitory components in the deuterated tryptamine sample. Reference to Fig. 3A indicates that, at low tryptamine concentrations (5 μM), there is still a sizeable KIE. At this concentration of substrate, the maximum concentration of a potential inhibitor would be <0.05 μM (based on >99% purity of the sample) and the AADH concentration is 1.3 μM. Clearly, therefore, reactions of AADH with potential inhibitor would be much less than stoichiometric. Additionally, the concentration dependence data for reactions with deuterated tryptamine fit well to the standard hyperbolic expression. If inhibitors were present, one might expect the hyperbolic dependence to break down at low substrate concentrations, as any potential inhibitor is effectively diluted out. Moreover, transients obtained with a 50:50 mixture of tryptamine and deuterated tryptamine (35 μM each) have a tangent at time 0 equal to the average of the tangents obtained with tryptamine (70 μM) and deuterated tryptamine (70 μM) alone. This observation again argues for the lack of an inhibitory component in deuterated tryptamine. Due to the large KIE, studies of the dependence of reaction rates on temperature were possible using deuterated tryptamine (Fig. 3, panel B). Studies with deuterated substrate revealed that ΔH_{D}^{‡}(tryptamine) = ΔH_{D}^{‡}(dopamine) = ΔH_{D}^{‡}(dopamine) (Table I). Even though detailed studies of the variation in reaction rate with temperature were not possible with tryptamine, our investiga-

### Figure 2

**Stopped-flow kinetic data for the reaction of AADH with dopamine and deuterated dopamine.**

**Panel A** shows the observed rate constant (k_{obs}) against dopamine concentration for the reaction with AADH. Closed circles represent dopamine, open circles represent deuterated dopamine ((HO)_{2}C_{6}H_{3}CH_{2}CD_{2}NH_{2}). For dopamine fitted to Equation 3, k_{d} = 132 ± 1 s⁻¹, K_{d} = 17.1 ± 0.8 μM. For deuterated dopamine fitted to Equation 3, k_{d} = 10.3 ± 0.1 s⁻¹, K_{d} = 12.1 ± 0.6 μM.

**Panel B** demonstrates temperature dependence and KIE data for the reaction of AADH with dopamine. Main panel, temperature dependence plots for AADH with dopamine (closed circles) and deuterated dopamine (open circles). ln(A_{D}) = 19.7 ± 0.3, ln(A_{H}) = 17.5 ± 0.3, ΔH_{C–H} = 50.9 ± 0.7 kJ mol⁻¹, ΔH_{C–D} = 51.6 ± 0.7 kJ mol⁻¹. Inset, plot of ln(KIE) versus 1/T. Rate constants are observed rate constants measured at 500 μM dopamine.

Zero. The value of the A_{H}^{‡}–A_{D}^{‡} ratio (9.4 ± 1.6) calculated from the intercepts of the plots is similar to that of the KIE (12.8 ± 0.2), indicating that the reaction proceeds predominantly by quantum tunneling. Observations similar to those discussed above for the temperature-dependent behavior of C–H bond breakage in AADH have been made during cleavage of a substrate C–H bond by the TTQ-dependent methylamine dehydrogenase (MADH) of *M. methylotrophus*. For MADH the data were interpreted in terms of vibrationally assisted tunneling of protons and deuterium through a fluctuating potential energy barrier (8). Our data for the reaction catalyzed by AADH indicate that this mechanismally similar enzyme catalyzes H-transfer by ground state tunneling of proton and deuterium and that tunneling is driven by the thermal motion.

The KIE data with dopamine indicate that H-transfer is not by the classical, over-the-barrier route and that quantum tunneling is the mechanism of transfer. Moreover, the data are inconsistent with rigid barrier models of H-tunneling since the vibrational assistance (as indicated by the nature of the temperature dependence plots) requires a fluctuating potential energy barrier for the transfer event. Vibrational assistance has been discussed for many years with regard to classical over-the-barrier reactions (35), but only relatively recently has this been applied to enzymatic quantum tunneling reactions. Temperature-independent KIEs with large enthalpies of activation can be interpreted in a number of ways. The dynamic component may reflect substrate vibrations, protein vibrations, or a combination of the two. Both Antoniou and Schwartz (36) and Borgis and Hynes (37) have provided theoretical descriptions of H-tunneling facilitated by vibrations in the substrate. Alternatively, Bruno and Bialek (38) have proposed that H-tunneling is facilitated by vibrations in the protein, and they have derived a relationship between the intrinsic KIE for C–H bond cleavage and temperature for tunneling reactions occurring from the vibrational ground state of the substrate. Importantly, in this treatment the KIE can adopt values below the “semiclassical” limit of 7 (at 25 °C); values in this limit and in systems consistent with tunnelling have recently been obtained experimentally for some enzymes (17, 22). A more general treatment of enzymatic H-tunneling involving coupling between the tunneling modes and the environment and a fluctuating barrier has been described recently by Kuznetsov and Ulstrup (39). Although we acknowledge that the degree and nature of H-tunneling in enzymatic systems is model-dependent, we feel our data are most consistent with the model proposed by Bruno and Bialek. In particular, the lack of an isotope effect on the activation enthalpy for H- and D-transfer suggests to us that normal vibrational modes of the substrate alone are not the major component of the vibrational assistance observed with AADH. We envisage this vibrational assistance comes predominantly from vibrational modes in the enzyme.

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**Fig. 3.** Stopped-flow kinetic data for the reaction of AADH with tryptamine and deuterated tryptamine. Panel A, plot of observed rate constant ($k_{\text{obs}}$) against [tryptamine] and [deuterated tryptamine]. Reactions were performed in 10 mM BisTris propane buffer, pH 7.5, at 4 °C. Transients were measured at 456 nm; [AADH] = 3. 

Closed circles—Equation 3, $A^{\text{H}}A^{\text{D}}$ (tryptamine--$\text{HCl}$). For tryptamine fitted to Equation 3, $k_0 = 503 \pm 5$ s$^{-1}$, $K_0 = 4.5 \pm 0.3$ µM. For deuterated tryptamine fitted to Equation 3, $k_0 = 9.25 \pm 0.07$ s$^{-1}$, $K_0 < 4$ µM (value is too small to measure using the stopped-flow technique). Panel B, temperature dependence plot for reaction of AADH with deuterated tryptamine. ln($k_{\text{obs}}$) = 19.8 ± 0.5, $\Delta H_{\text{C-D}}$ = 53.5 ± 1.2 kJ mol$^{-1}$. Rate constants are observed rate constants measured at 180 µM deuterated tryptamine.

with deuterated tryptamine suggest that vibrationally assisted tunneling (probably from the vibrational ground state of the substrate in accordance with the model of Bruno and Bialek) is the mechanism of H-transfer with this substrate. This follows, since either tunneling from an excited state or transfer over-the-barrier would likely manifest itself as $\Delta H_{\text{C-D}}^{\text{tryptamine}} > \Delta H_{\text{C-D}}^{\text{dopamine}}$. Comparable studies were also performed using the slow substrate benzylamine (Fig. 4, panels A and B). In this case, the KIE (4.8 ± 0.2) is reduced compared with dopamine (KIE = 12.9 ± 0.2) and tryptamine (KIE = 54.7 ± 1.0), but temperature dependence studies suggest that vibrationally assisted tunneling occurs from the vibrational ground state of the substrate. In particular, the temperature dependence plots (Fig. 4, panel B) illustrate the parallel nature of the C–H and C–D data ($\Delta H_{\text{benzylamine}}^{\text{C-H}}$, $\Delta H_{\text{benzylamine}}^{\text{C-D}}$), and the $A^{\text{H}}A^{\text{D}}$ ratio is comparable with the KIE (Table 1). indicating that the reaction proceeds predominantly by quantum tunneling. With benzylamine, however, the enthalpy of activation is higher than that seen with dopamine and tryptamine. The data thus suggest that there is a larger ener-

getic cost in deforming the enzyme-substrate iminoquinone intermediate with this substrate, which is required to facilitate H-tunneling by barrier compression and equalization of the reactant and product energies.

**Variation in KIE as a Function of Barrier Width**—Our studies with AADH suggest that the cleavage of the substrate C–H bond with slow (benzylamine) and fast (dopamine and tryptamine) substrates occurs by a vibrationally assisted tunneling mechanism. This tunneling process is consistent with KIE values ranging from 4.8 (benzylamine) to 54.7 (tryptamine), and an explanation for this variation in KIE needs to be sought.

Considering the effect of barrier width (and shape) on wave function decay for the protium and deuterium nuclei, one can develop an explanation for the variation in intrinsic KIE in reactions of different substrates. Effects of barrier shape on quantum tunneling reactions have been discussed previously (e.g. Ref. 40). These analyses have invoked static potential energy barriers and discussed idealized barrier shapes (e.g. truncated paraboloid). Although a fluctuating potential energy barrier is consistent with our experimental observations, we have discussed previously (8, 23, 24) that the tunneling event can be visualized as a two-step process. The first step is dynamical in nature and is required to activate the enzyme-substrate complex by thermal vibration. In essence, it leads to a crossing over of the potential energy surfaces for the enzyme-substrate and enzyme-product complexes. The second step is a quantum tunneling component, which occurs only when the activated complex is populated (i.e. at the crossing point of the enzyme-substrate and enzyme-product potential energy curves). Thus, although we have established experimentally that the potential energy barrier to the reaction is fluctuating, when the geometry is compatible with quantum tunneling (i.e. at intersection point of the potential energy surfaces of the enzyme-substrate and enzyme-product complexes), the tunneling barrier can be treated as being rigid for the lifetime of the tunneling event. In the discussion below, therefore, we have used rigid barrier depictions of H-tunneling to predict the effect of barrier shape and width on the value of the KIE obtained.

Factors that enhance tunneling are a small particle mass (increased de Broglie wavelength) and a small area under the potential energy barrier, but the barrier needs to be sufficiently high to favor tunneling, rather than classical over-the-barrier, reactions; thus, high, narrow barriers are particularly favor-

| AADH parameter | Tryptamine | Dopamine | Benzylamine |
|----------------|------------|----------|-------------|
| $k_{\text{lim}}^{\text{C-H}}$ (s$^{-1}$) | 503 ± 5 | 132 ± 1.0 | 1.81 ± 0.02 |
| $k_{\text{lim}}^{\text{C-D}}$ (s$^{-1}$) | 9.25 ± 0.07 | 10.3 ± 0.07 | 0.38 ± 0.01 |
| KIE | 54.7 ± 1.0 | 12.9 ± 0.2 | 4.8 ± 0.2 |
| $A^{\text{H}}A^{\text{D}}$ | ND | 9.4 ± 1.6 | 3.7 ± 2.0 |
| $\Delta H_{\text{C-H}}^{\text{D}}$ (kJ mol$^{-1}$) | ND | 50.9 ± 0.7 | 68.1 ± 1.4 |
| $\Delta A^{\text{H}}A^{\text{D}}^{\text{D}}$ (kJ mol$^{-1}$) | 53.5 ± 1.2 | 51.6 ± 0.7 | 67.1 ± 0.9 |

| MAddH parameter | Methyamine$^a$ | Ethanolamine |
|----------------|-------------|-------------|
| $k_{\text{lim}}^{\text{C-H}}$ (s$^{-1}$) | 175 ± 4 | 14.1 ± 0.07 |
| $k_{\text{lim}}^{\text{C-D}}$ (s$^{-1}$) | 10.4 ± 0.1 | 0.96 ± 0.01 |
| KIE | 16.8 ± 0.5 | 14.7 ± 0.2 |
| $A^{\text{H}}A^{\text{D}}$ | 13.3 | 0.57 ± 1.71 |
| $\Delta H_{\text{C-H}}^{\text{D}}$ (kJ mol$^{-1}$) | 44.6 ± 0.5 | 43.5 ± 0.6 |
| $\Delta H_{\text{C-D}}^{\text{D}}$ (kJ mol$^{-1}$) | 45.0 ± 0.5 | 51.9 ± 1.1 |

$^a$ Limiting rates of TTQ reduction and KIEs are quoted for data obtained at 25 °C, except for the tryptamine data, which was obtained at 4 °C. With tryptamine, H-transfer rates are too fast to measure at higher temperatures using the stopped-flow technique.

$^b$ ND, not determined.

$^c$ Data taken from Ref. 8.
Barrier Shape in Enzyme Reactions

been discussed in detail previously in Ref. 38). The amplitude of the wave function (kinetic energy $E$) decreases exponentially within the barrier, and the tunneling rate, $k$, is related to the mass of the tunneling particle, $m$, by the following relationship.

$$k = \exp(-2l \sqrt{2m(V - E)}/h)$$

(Eq. 7)

Inspection of Equation 7 reveals that, for a nucleus of a given mass, $k$ decays exponentially with increased tunneling distance and/or increased barrier height. Thus, given the observed rates (Table I), a rectangular barrier predicts that tunneling from tryptamine occurs over a shorter distance and/or a lower barrier than tunneling from dopamine, which in turn occurs over a shorter distance and/or through a lower barrier than tunneling from benzylamine. The KIE can be obtained from Equation 7 as follows.

$$\text{KIE} = \frac{k_H}{k_D} = \exp(\frac{-2l \sqrt{2m(V - E_H)}/h} - \frac{-2l \sqrt{2m(V - E_D)}/h})$$

(Eq. 8)

In Equation 8, $k_H$ and $k_D$, $m_H$ and $m_D$, and $E_H$ and $E_D$ are the tunneling rates for, masses, and zero point energies of D and H, respectively. Equation 8 reveals that the KIE increases exponentially with the tunneling distance and/or barrier height. Thus, for the observed KIEs (Table I), a rectangular barrier predicts that tunneling from tryptamine occurs over a longer distance and/or through a higher barrier than tunneling from dopamine, which in turn occurs over a longer distance and/or through a higher barrier than from benzylamine. This discrepancy between the relative tunneling distances and/or barrier heights derived from $k$ and KIE values (Fig. 5, panel C) clearly illustrates that the rectangular barrier is not the correct shape for the reaction of AADH with the substrates tryptamine, dopamine, and benzylamine.

A truncated parabolic potential energy barrier provides an alternative barrier shape (40) (Fig. 5, panel B). The potential, $V(x)$, can be described by Equation 9.

$$V(x) = V \left(1 - \frac{x^2}{a^2}\right) \quad \text{for } |x| < a$$

$$= 0 \quad \text{for } |x| \geq a$$

(Eq. 9)

$V$ is the height of the barrier and $2a$ is the maximum width of the barrier. The tunneling rate, $k$, is related to the mass of the tunneling particle, $m$, and the tunneling distance, $2l$, by the following relationship.

$$k = \exp \left[\frac{2}{h} \int_{-a}^{a} \sqrt{2m(V(x) - E)} dx\right]$$

(Eq. 6)

In Equation 6, $S$ is known as the Wentzel-Kramers-Brillouin action, $V(x)$ is the potential energy barrier, and $E$ is the zero point energy of the H nucleus.

To illustrate the effect of H- versus D-tunneling, consider first the simplest potential energy barrier: a rectangular barrier (height $V$, width $l$; Fig. 5, panel A); the possible role of a rectangular barrier in vibrationally assisted tunneling has

$$E = V \left(1 - \frac{l^2}{a^2}\right)$$

$$l = a \sqrt{\frac{E}{V}}$$

(Eq. 11)

Substituting Equation 11 in Equation 10 gives Equation 12.
As with the rectangular barrier, given the observed rates (Table I), a truncated parabolic barrier predicts that tunneling from tryptamine occurs through a narrower barrier than tunneling from dopamine, which in turn occurs through a narrower barrier than tunneling from benzylamine. Additionally, it can be shown numerically from Equation 12 that, for a fixed barrier width (i.e., fixed value of $a$), the barrier height must increase from tryptamine to dopamine to benzylamine to explain the trend in the experimental rates. The KIE can be obtained from Equation 12, giving Equation 13.

As with the rectangular barrier, Equation 13 reveals that, for a truncated parabolic barrier, the KIE increases exponentially with the barrier width, thus predicting that tunneling from tryptamine occurs over a longer distance than tunneling from dopamine, which in turn occurs over a longer distance than from benzylamine. Additionally, it can be shown numerically from Equation 13 that, for a fixed barrier width, increasing the

$$k = \exp \left[ -\frac{a \sqrt{2m}}{\hbar} \left( \sqrt{E - V} \sqrt{\frac{E}{V}} + \sqrt{V \sin^{-1}} \sqrt{1 - \frac{E}{V}} \right) \right] \quad \text{(Eq. 12)}$$

$$\text{KIE} = \exp \left[ \frac{a \sqrt{2m}}{\hbar} \left( \sqrt{rac{E_0 - E_d}{V}} - \sqrt{m_H} \sqrt{E_H - E_d} \right) \right. \right.$$  

$$\left. + \sqrt{V \left( \sqrt{m_D} \sin^{-1} \sqrt{1 - \frac{E_0}{V}} \right) \sqrt{m_H} \sin^{-1} \sqrt{1 - \frac{E_H}{V}}} \right) \quad \text{(Eq. 13)}$$

As with the rectangular barrier, the KIE increases exponentially with the barrier width, thus predicting that tunneling from tryptamine occurs over a longer distance than tunneling from dopamine, which in turn occurs over a longer distance than from benzylamine. Additionally, it can be shown numerically from Equation 13 that, for a fixed barrier width, increasing the
barrier height increases the predicted KIE. Again, this discrepancy between the relative tunneling distances and/or barrier heights derived from $k$ and KIE values (Fig. 5, panel D) clearly illustrates that the truncated parabolic barrier is not the correct shape for the reaction of AADH with the substrates tryptamine, dopamine, and benzylamine.

Rectangular and parabolic barriers are appropriate (albeit crude) depictions of the potential energy surface near the top of the barrier. Other barrier shapes have been suggested, e.g., Eckart and Gaussian (40). It could be argued that Eckart and Gaussian (along with other shapes) barriers are more realistic depictions of the potential energy surface when tunneling is from the vibrational ground state of the substrate. However, our experimental data suggest that a “nonstandard” barrier shape may be required to explain the experimental observations. One possible shape that is consistent with the trends in experimental rates and KIEs is shown in Fig. 5 (panel E; note that, in this schematic, for simplicity we have assumed that it is only the barrier width that changes, and not the height, although in practice both will likely vary). The advantage of this barrier over those discussed above is that it has a concave shoulder occurring just below where H tunnels, which provides a possible explanation for the relatively high rates and high KIE observed for tryptamine. The relatively fast rates for tryptamine can be explained by tunneling occurring through a relatively narrow barrier compared with dopamine and benzylamine. The large KIE for tryptamine can be explained by D tunneling through a shoulder below the narrow part of the barrier, whereas H tunnels above this shoulder through the narrow part of the barrier; thus, D would tunnel significantly further than H. The narrowest part of the barrier is positioned closer to the reactants than to the products, as this part of the potential energy surface is likely to correspond to the point at which the C–H and C–D bonds are broken. As the enzyme-catalyzed reaction becomes less efficient, one possible scenario is that the narrowest part of the barrier could become progressively broader and, as a result, the shoulder become increasingly less pronounced. Thus, with dopamine (Fig. 5, panel E), the shoulder could still be present but H, and to a much lesser extent D, would have further to tunnel than with tryptamine. This would manifest itself in (i) slower rates and (ii) a lower KIE for dopamine than tryptamine. With benzylamine, the shoulder could be less pronounced still. Thus, although the integrated area under the barrier has become larger and this would be expected to result in higher KIEs, this is more than offset by the more similar tunneling distances for H and D and manifests itself in (i) the slowest rates and (ii) the lowest KIE of the three AADH substrates.

Although the barrier shape depicted in Fig. 5 (panel E) is consistent with experimental data, it is nevertheless just one possibility; the true nature of the barrier shape for AADH (and MADH) remains to be established. These barriers are currently being investigated using quantum mechanical/molecular mechanical computational methods.

Reaction of MADH with the Slow Substrate Ethanolamine—We have also probed the effect of using slow substrates on the quantum tunneling reaction catalyzed by MADH. The dependence of the rate of TTQ reduction on [ ethanolamine] is shown in Fig. 6 and the associated parameters in Table I. The limiting rates of TTQ reduction are approximately 1 order of magnitude slower than those observed with methylamine (Table I). Temperature dependence studies of TTQ reduction with ethanolamine (Fig. 6; Table I) indicate that the mechanism of slow substrate oxidation by MADH is different to that of AADH. With AADH, temperature-independent KIEs were observed with dopamine and benzylamine. In reactions of MADH with ethanolamine, however, the KIE is temperature-dependent, $\Delta H^{EH}$ethanolamine > $\Delta H^{EH}$ethanolamine and $\Delta H^{D}$< 1. These parameters suggest that tunneling does occur with ethanolamine, but that tunneling with the deuterated substrate is from part way up the potential energy barrier (42). Partial ascent of the barrier with deuterated substrate by thermal activation will reduce the barrier width to the point where nuclear tunneling becomes favorable. The extra thermal energy required for this process is reflected in the relatively large value of $\Delta H^{D}$ethanolamine, compared with $\Delta H^{D}$ethanolamine. In all likelihood, protium transfer from ethanolamine to MADH is from the ground state since $\Delta H^{EH}$ethanolamine = $\Delta H^{EH}$methylamine (Table I). The decrease in rate observed with ethanolamine is likely due to an increase in barrier width/height.

Conclusions—TTQ-dependent amine dehydrogenases catalyze C–H bond cleavage by a vibrationally assisted quantum tunneling mechanism. We infer that the size of the KIE with alternative substrates varies with barrier width, as expected.
for a pure tunneling reaction. Experimental data are inconsistent with idealized rectangular and truncated parabolic energy barriers, but are consistent with more complex barrier shapes for C–H bond cleavage. Deuterium transfer from a slow substrate (ethanolamine) to MADH requires partial ascent of the potential energy surface to reduce the tunneling pathway distance.

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