Enzymatic H-Transfer Requires Vibration-Driven Extreme Tunneling†

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ABSTRACT: Enzymatic breakage of the substrate C−H bond by *Methylophilus methyltrophus* (sp. W_3A_1) methylamine dehydrogenase (MADH) has been studied by stopped-flow spectroscopy. The rate of reduction of the tryptophan tryptophylquinone (TTQ) cofactor has a large kinetic isotope effect (KIE = 16.8 ± 0.5), and the KIE is independent of temperature. Analysis of the temperature dependence of C−H bond breakage revealed that extreme (ground state) quantum tunneling is responsible for the transfer of the hydrogen nucleus. Reaction rates are strongly dependent on temperature, indicating thermally induced, vibrational motion drives the H-transfer reaction. The data provide direct experimental evidence for enzymatic bond breakage by extreme tunneling driven by vibrational motion of the protein scaffold. The results demonstrate that classical transition state theory and its tunneling derivatives do not adequately describe this enzymatic reaction.

MADH[1] from *Methylophilus methylotrophus* (sp. W_3A_1) catalyzes the oxidative demethylation of methylamine to formaldehyde and ammonia *(1)*:

$$CH_3NH_2 + H_2O \rightarrow CH_2O + NH_3 + 2H^+ + 2e^-$$

Electrons derived from substrate are transferred to a TTQ cofactor *(2)* in the reductive half-reaction sequence (Figure 1). In the oxidative half-reaction, electrons are transferred to the blue copper protein amicyanin and a *c*-type cytochrome *(3−5).* During the reductive half-reaction, one of the C−H bonds of methylamine is cleaved. The reaction is initiated by nucleophilic attack of the substrate nitrogen on the quinone carbon to form a carbinolamine intermediate *(6),* followed by loss of water to produce the iminoquinone (Figure 1). An active-site base *(7)* then abstracts a proton from the iminoquinone intermediate to form a carbanion with concomitant reduction of the TTQ cofactor as shown (Figure 1). This reaction sequence is just one of many chemical mechanisms employed by enzymes for the oxidation of amine substrates—other enzymes catalyzing similar transformations include the flavoproteins trimethylamine dehydrogenase *(8)* and sarcosine oxidases *(9),* and the copper-containing amine oxidases that utilize topa quinone as the redox cofactor *(10).* A common and major problem faced by these enzymes is the perceived difficulty in cleaving a stable substrate C−H bond, which potentially gives rise to large activation energies when modeled using the classical TST approach to enzyme catalysis *(11).*

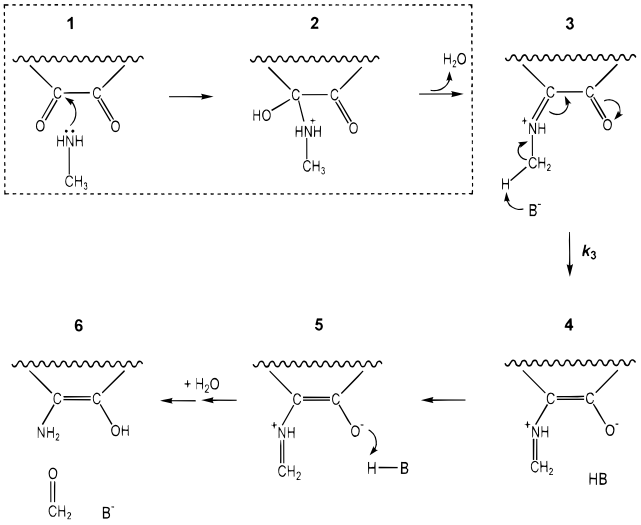

FIGURE 1: Mechanism for the reductive half-reaction of MADH. Steps enclosed in the hatched box represent binding steps [rate constants k_1 (forward reaction) and k_2 (reverse reaction)].

The majority of enzymes catalyze the transfer of hydrogen in some form—examples include proton movements (acid/base catalysis), hydride transfer (redox catalysis), and radical transfer (homolysis of a substrate-H bond). Correct theoretical treatment of these reactions is central to our understanding of biological catalysis. Hitherto, the overwhelming trend has been to model them using the classical formulations of transition state theory (TST) *(11).* However, the pioneering work of Klinman and co-workers has shown small deviations from classical behavior *(12−15),* and they have interpreted these as indicating transfer by quantum mechanical tunneling. They have also studied one system where large deviations from classical behavior are observed *(16),* and attributed this to extreme tunneling (i.e., tunneling of both protium and deuterium). The major prediction from these data that tunneling becomes more prominent as the apparent activation energy decreases *(16)* holds for those enzymes studied by

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[1] Abbreviations: MADH, methylamine dehydrogenase; TST, transition state theory; KIE, kinetic isotope effect; TTQ, tryptophan tryptophylquinone.

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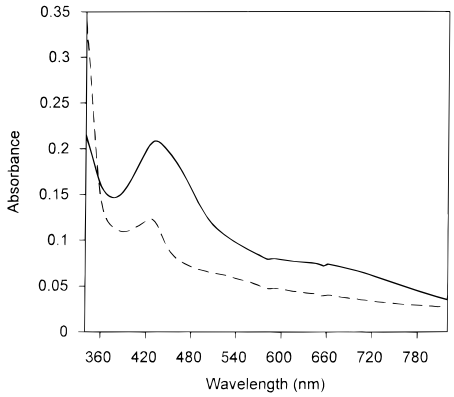


FIGURE 2: Absorbance spectra for $MADH_{ox}$ (solid line) and the substrate-reduced form of MADH (hatched line). Enzyme was contained in 10 mM potassium phosphate buffer, pH 7.5. Enzyme concentration is 8 μM. Reduction was achieved by the addition of methylamine (final concentration 1.3 mM).

Klinman and co-workers. However, our studies presented here show that this correlation breaks down for the catalytic breakage of a C−H bond by the enzyme MADH, and that this shortcoming is a direct result of invoking a static [Eyring-like *(17)*] rather than fluctuating [Kramers-like *(18)*] potential energy barrier. We selected MADH as a model system for our studies because (i) the mechanism of TTQ cofactor reduction is well characterized [Figure 1; *(19)*], (ii) analysis by stopped-flow techniques enables direct measurement of the rate of C−H bond breakage *(20)*, and (iii) C−H bond breakage by MADH is associated with an inflated KIE (>7), suggestive of nuclear tunneling *(20)*. In this paper, we demonstrate that MADH catalyzes breakage of the C−H bond of methylamine by a vibration-driven extreme (i.e., ground state) tunneling mechanism. Extreme tunneling avoids transfer by the classical over-the-barrier route, and may be a general mechanism for the breakage of stable C−H bonds by enzymes.

EXPERIMENTAL PROCEDURES

MADH was purified from *Methylophilus methylotrophus* (sp. W_3A_1) as described *(21)*. Following reoxidation of the isolated enzyme with 100-fold excess of ferricyanide *(21)*, 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 *(21)*. Rapid reaction kinetic experiments were performed using an Applied Photophysics SX.18MV stopped-flow reaction analyzer. Time-dependent reduction of MADH by methylamine was measured at 440 nm (reduction of the iminoquinone intermediate; Figure 2) in 10 mM potassium phosphate buffer, pH 7.5, and data were analyzed using nonlinear least-squares regression analysis on an Archemedes 410-1 microcomputer using Spectrakinetic software (Applied Photophysics). In stopped-flow studies, the enzyme concentration was 1.5 μM, and substrate was always at least 10 times this concentration to ensure pseudo-first-order conditions. For each substrate concentration, at least four replicate measurements were collected and averaged. Substrate-reduced MADH is stable to reoxidation by molecular oxygen, negating the use of anaerobic conditions. For reactions under more viscous conditions, 30% glycerol was included in all solutions.

As reported previously *(19, 20)*, the absorbance change at 440 nm was found to be monophasic, with a single rate constant obtained from fits of the data to eq 1:

$$A_{440} = Ce^{-k_{obs}t} + b \quad (1)$$

C is a constant related to the initial absorbance, and b is an offset value to account for a nonzero base line. The observed rate constants were found to exhibit substrate dependence. Data from previous stopped-flow studies on the *Paracoccus denitrificans* enzyme *(20)* were fitted using the equation described by Hiromi *(22)*:

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

However, as seen for the *Paracoccus denitrficans* enzyme *(20)*, fits to this equation produce large KIEs for k_1 and k_2 (see below), which are much too large for secondary isotope effects. This brings into question the accuracy of eq 2 in the context of the chemical scheme described in Figure 1. Systematic deviations are seen when the data are fitted to the standard hyperbola (see Results). Although eq 2 is inappropriate in the low substrate concentration regime, it provides good estimates of the rate constant k_3 in the high substrate regime. The rate of TTQ reduction in temperature dependence studies was taken as the observed rate constant, k_{obs}, at saturating concentrations of substrate (500 μM).

RESULTS AND DISCUSSION

Previous workers have demonstrated an unusually high KIE for the reductive half-reaction of MADH using perdeuterated methylamine as substrate *(19, 20)*. Although suggestive of tunneling, a detailed thermodynamic analysis of H-transfer has not been reported for any MADH. Analysis of the reductive half-reaction of the *Methylophilus methylotrophus* enzyme at pH 7.5 confirmed that reduction of the TTQ cofactor was subject to an unusually high KIE (Figure 3). Like the *Paracoccus denitrificans* enzyme *(20)*, large KIEs were found for the rate constants k_1 and k_2 (KIEs are 6 ± 2 and 19 ± 12, respectively) when eq 2 was used to analyze the data. The rate constants k_1 and k_2 represent formation and decay of the iminoquinone intermediate (Figure 1), and it has been reported previously *(20)* that the large KIEs on these constants reflect a mechanism involving hyperconjugation of the methyl group of the substrate during reversible imine formation. However, these isotope effects are much too large to be attributable to secondary KIEs. The data suggest that eq 2 is inappropriate for describing the reduction of TTQ in MADH, but equally the standard hyperbolic expression leads to some systematic deviation in data fitting (Figure 3). Equation 2, however, provides for a reliable estimate of k_3 (the rate of TTQ reduction at saturating substrate). Importantly, an unusually large KIE (16.8 ± 0.5) is found for the rate constant k_3, which describes the rate of proton abstraction from the iminoquinone intermediate and concerted reduction of TTQ (Figure 1). This large KIE indicates that the chemical step is rate-limiting.[2]

Large KIEs have been reported for several enzymes, especially those cleaving C−H bonds [e.g., *(23)*], but these values alone do not provide evidence for a tunneling reaction

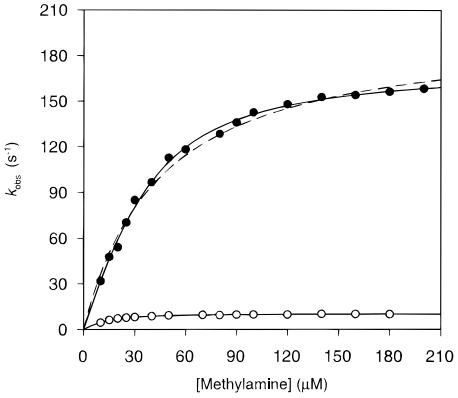


FIGURE 3: Plot of observed rate constant (k_{obs}) against methylamine concentration for the reduction of the TTQ cofactor in MADH. Reactions were perfomed in 10 mM potassium phosphate buffer, pH 7.5, at 25 °C. The fits shown are to eq 2 (solid line) or to the standard hyperbolic expression (dashed line). Closed circles, methylamine; open circles, perdeuterated methylamine. For methylamine fitted to eq 2, $k_1 = 5.4 \times 10^6 \pm 0.8 \times 10^6$ M^{-1} s^{-1}, $k_2 = 95 \pm 31$ s^{-1}, $k_3 = 175 \pm 4$ s^{-1}; for perdeuterated methylamine fitted to eq 2, $k_1 = 0.85 \times 10^6 \pm 0.12 \times 10^6$ M^{-1} s^{-1}, $k_2 = 4.9 \pm 1.3$ s^{-1}, $k_3 = 10.4 \pm 0.1$ s^{-1}. For methylamine fitted to the standard hyperbola, $k_3 = 198 \pm 5$ s^{-1}, $K_d = 44 \pm 3$ μM; for perdeuterated methylamine fitted to the standard hyperbola, $k_3 = 11.0 \pm 0.1$ s^{-1}, $K_d = 11.6 \pm 0.7$ μM. For clarity, the hyperbolic fit is not shown fitted to the perdeuterated methylamine data. However, systematic deviations in the fits to both methylamine and perdeuterated methylamine data were observed.

(24). Traditionally, static potential energy barrier systems incorporating tunneling corrections have been used to model these reactions *(25)*. By using the Arrhenius equation, several criteria have been established to identify tunneling reactions *(15, 26, 27)*. These criteria include (i) large differences in the activation energies for protium and deuterium transfer ($\Delta\Delta E_a > 5.4$ kJ mol^{-1}, i.e., larger than the difference in the zero-point vibrational energies for the C−H and C−D bonds) and (ii) an Arrhenius preexponential factor ratio $A^H:A^D < 1$. For extreme tunneling, $A^H:A^D > 1 \approx$ KIE, $\Delta\Delta E_a \approx 0$, and temperature-independent KIEs are predicted *(28)* using the static barrier approach (Figure 4). To date, most enzymes in which tunneling has been demonstrated persuasively operate within regime II of Figure 4. In one case, extensive tunneling (regime III) has been demonstrated for lipoxygenase *(16)*.

To ascertain the mechanism of C−H bond breakage in MADH, we have investigated the temperature dependence and solvent viscosity dependence of the proton abstraction step using methylamine and perdeuterated methylamine as substrate. In these experiments, saturating concentrations (500 μM) of methylamine were used, and buffer conditions were identical to those described for the analysis of the substrate dependence on k_{obs}. Given the concerted nature of C−H bond breakage and TTQ reduction, it should be emphasized that the observed rates of TTQ reduction report directly on C−H bond breakage. The relatively slow rate of C−H bond breakage ($k_3 = 175 \pm 4$ s^{-1} at 25 °C, pH 7.5) and the inflated KIE make it likely that the determined KIE is intrinsic in temperature-dependent studies using 500 μM methylamine (i.e., a regime in which the methylamine concentration is saturating).

[2] Since an *upper* value for secondary KIEs is 1.36 [*(35)*; i.e., 8% of the observed KIE], secondary effects should not compromise our conclusions.

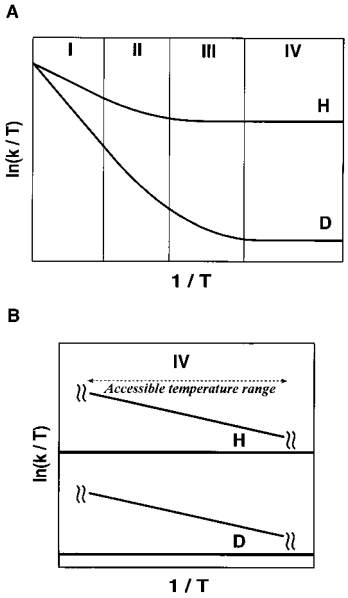


FIGURE 4: General relationship describing schematically how the rate of hydrogen transfer depends on temperature. Panel A: a static potential energy barrier. Regime I: classical relationship, conforms to the unimolecular rate law (eqs 3 and 4), characterized by large ΔH and $A'^H:A'^D = 1$. Regime II: more extensive tunneling of protium compared with deuterium, gives rise to inflated KIEs (>7) and $A'^H:A'^D < 1$. Regime III: characterized by more extensive tunneling with $A'^H:A'^D$ ratios difficult to predict. Regime IV: extreme tunneling, predicts $\Delta\Delta H \approx 0$ and $A'^H:A'^D =$ KIE. Panel B: comparison of temperature dependence in regime IV for a static (horizontal line) and fluctuating (line with negative gradient) barrier in the accessible temperature range. The relationship can be extrapolated to lower temperatures for the static barrier. Complex temperature dependencies prevent extrapolation of the relationship for the fluctuating barrier beyond the experimentally accessible temperature range.

Previous studies of H-tunneling in enzymes have been analyzed in terms of the phenomenological Arrhenius plot (e.g., *16*)—i.e., a plot of ln(k) vs 1/T. Although the Arrhenius plot appears linear in the accessible temperature range, it is in fact curved and asymptotically approaches infinity at high temperatures. Apparent linearity in the accessible temperature range does not compromise data analysis in this regime, but temperature dependence studies should strictly be analyzed in terms of the equation describing a unimolecular reaction. The temperature dependence for a unimolecular rate constant is given by eq 3

$$k = \frac{k_B}{h} T e^{-\Delta G^{\ddagger}/RT} = \frac{k_B}{h} T e^{-\Delta H^{\ddagger}/RT} e^{\Delta S^{\ddagger}/R} \qquad (3)$$

where k_B and h are the Boltzmann and Planck constants, respectively. A convenient way of plotting the temperature dependence of a unimolecular reaction is to use eq 4

$$\ln(k/T) = \ln(k_B/h) + \Delta S^{\ddagger}/R - \Delta H^{\ddagger}/RT \qquad (4)$$

The activation parameter $\Delta H^{\ddagger}$ is calculated from the slope of the plot. $\Delta S^{\ddagger}$ is calculated by extrapolation to the ordinate axis, and $\Delta G^{\ddagger}$ is calculated directly from eq 3. The use of eq 4 in plotting the temperature dependence of a unimolecular reaction is preferred over the use of the classical Arrhenius plot, and for this reason the former was used in temperature

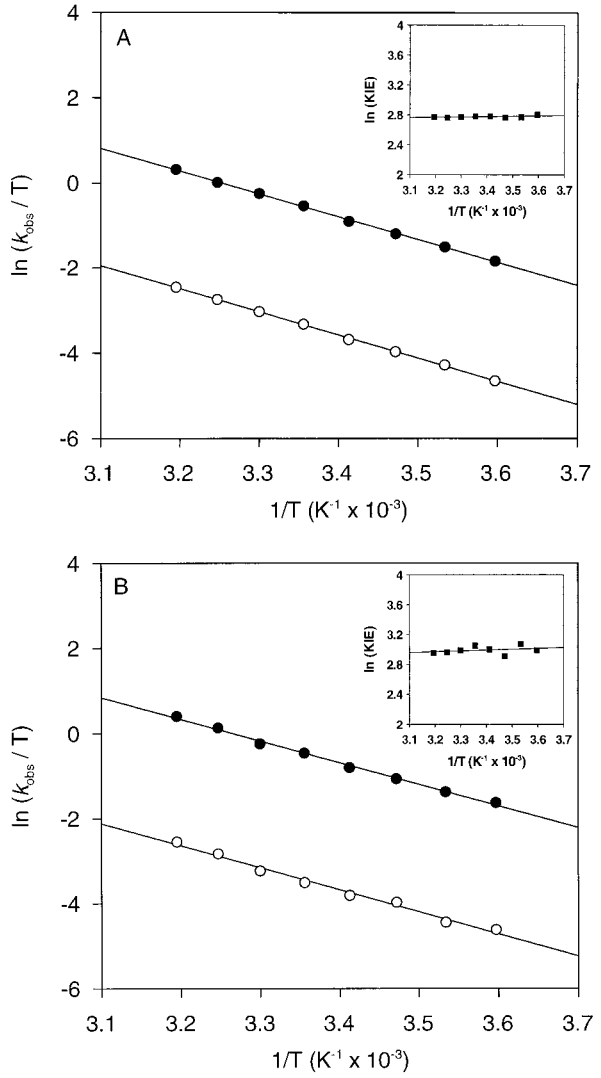


FIGURE 5: Temperature dependence and KIE data for MADH. (A) Main panel: temperature dependence plots for MADH with methylamine (closed circles) and perdeuterated methylamine (open circles) as substrates in the absence of glycerol. $\ln(A'^{H}) = 17.5 \pm 0.2$, $\ln(A'^{D}) = 14.9 \pm 0.2$, $\Delta H^{H} = 44.6 \pm 0.5$ kJ mol^{-1}, $\Delta H^{D} = 45.0 \pm 0.5$ kJ mol^{-1}. Inset: plot of ln(KIE) versus $1/T$. Rate constants are observed rate constants measured at 500 μM substrate. Fitting to the Arrhenius equation (plot not shown) yields the following parameter values: $\ln(A^{H}) = 24.1 \pm 0.2$, $\ln(A^{D}) = 21.5 \pm 0.2$, $\Delta E_a^{H} = 47.1 \pm 0.5$ kJ mol^{-1}, $\Delta E_a^{D} = 47.5 \pm 0.5$ kJ mol^{-1}. (B) Main panel: temperature dependence plots for MADH with methylamine (closed circles) and perdeuterated methylamine (open circles) as substrates in the presence of 30% glycerol. $\ln(A'^{H}) = 16.6 \pm 0.5$, $\ln(A'^{D}) = 14.0 \pm 0.7$, $\Delta H^{H} = 42.2 \pm 1.1$ kJ mol^{-1}, $\Delta H^{D} = 43.2 \pm 1.8$ kJ mol^{-1}. Inset: plot of ln(KIE) versus $1/T$. Rate constants are observed rate constants measured at 500 μM substrate. Fitting to the Arrhenius equation (plot not shown) yields the following parameter values: $\ln(A^{H}) = 23.5 \pm 0.5$, $\ln(A^{D}) = 20.6 \pm 0.7$, $\Delta E_a^{H} = 44.6 \pm 1.1$ kJ mol^{-1}, $\Delta E_a^{D} = 45.5 \pm 1.8$ kJ mol^{-1}.

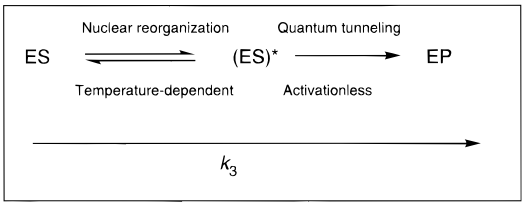
dependence studies of the MADH reaction (Figure 5). A necessary consequence of using eq 4 is the need to define explicitly the meaning of values obtained from such plots. Use of the Arrhenius plot has led to the development of criteria to indicate tunneling based on the values for $\Delta\Delta E_a$ and the A^{H}:A^{D} ratio (calculated from the intercepts of the Arrhenius plot for protium and deuterium substrates). The corresponding parameters calculated from the slopes and intercepts of plots using eq 4 are $\Delta\Delta H^{\ddagger}$ and A'^{H}:A'^{D} (the prime is used to distinguish this ratio from the A^{H}:A^{D} ratio calculated from the classical Arrhenius plot).

Analysis by eq 4 of the reaction catalyzed by MADH for both substrates revealed that the KIE is independent of temperature and that the difference in the enthalpy of activation for protium versus deuterium transfer ($\Delta\Delta H^{\ddagger} = \Delta H^{\ddagger D} - \Delta H^{\ddagger H} = 0.4 \pm 1.0$ kJ mol^{-1}) is essentially zero (Figure 5). Importantly, the A'^{H}:A'^{D} ratio determined from the intercepts of the plots is 13.3. As predicted for extreme tunneling in regime IV *(16)*, the A'^{H}:A'^{D} ratio is similar to the observed KIE (16.8 ± 0.5) indicative of an "activationless" reaction—i.e., ground-state quantum tunneling ($\Delta\Delta H^{\ddagger} = 0$). Paradoxically, however, the experimentally determined activation energies are clearly nonzero—$\Delta H^{\ddagger H} = 44.6 \pm 0.5$ kJ mol^{-1} and $\Delta H^{\ddagger D} = 45.0 \pm 0.5$ kJ mol^{-1}, and, as this would predict, reaction rates with methylamine and perdeuterated methylamine are highly dependent on temperature. This paradox can be resolved by invoking a fluctuating, temperature-dependent, potential energy surface that drives proton abstraction by quantum tunneling. This is consistent with a number of theoretical studies *(29−33)* which have suggested a fluctuating potential energy surface resulting from fluctuations in the protein, fluctuations in the substrate, or fluctuations in both the protein and substrate. In these theoretical treatments, the only means of barrier crossing is by quantum tunneling. In the case of MADH, it is the thermal fluctuation of the protein that predominantly drives H-transfer. Fluctuations of the substrate do not contribute substantially to the H-transfer reaction—these would give rise to different, nonzero values of the activation energies for protium and deuterium transfer, which is not supported by experiment.

For MADH, the static barrier approach to H-tunneling (Figure 4) is clearly inappropriate. For MADH, region IV of the relationship illustrated in Figure 4—and in the general case regions I, II, and III—needs to be modified to account for a fluctuating energy barrier. In the accessible temperature range, the data for MADH yield apparent linear plots with a sizable enthalpy term. This contrasts with the temperature-independent behavior seen in region IV for the static barrier relationship (Figure 4). Unlike for the static barrier (region IV), the apparent linearity seen in the accessible temperature range for MADH should not extend to lower temperatures. At low temperatures, nuclear vibrations will be frozen, thus preventing distortion of the nuclear scaffold into geometries compatible with nuclear tunneling. Over a large temperature range, complex temperature dependencies on the reaction rate are expected, and these are difficult to predict. Such complexities have been discussed in relation to theoretical treatments of H-tunneling in condensed media *(32)*.

Prior to the tunneling event, the MADH H-transfer reaction requires a temperature-dependent nuclear component that reflects reorganization of the protein scaffold to a geometry compatible with H-transfer by activationless ground-state tunneling (i.e., moving along the reactant curve to the intersection point of the reactant and product potential energy curves). This is summarized in Scheme 1, where ES is the iminoquinone adduct form of MADH, (ES)* is the activated geometry of the iminoquinone adduct form, and EP is the product of C−H bond breakage. In principle, this scheme leads to three possible regimes in which either (i) nuclear reorganization is rate-limiting, (ii) quantum tunneling is rate-

Scheme 1



limiting, or (iii) both these factors contribute to the observed rate *(29)*. Nuclear reorganization is not rate-limiting since this would lead to a KIE of unity—contrary to experimental findings. In the quantum tunneling-limiting regime, the KIE is predicted to be neither dependent on solvent viscosity nor equal to unity. Alternatively, when both nuclear reorganization and quantum tunneling contribute to the observed rate, the KIE is viscosity dependent—as viscosity increases, the KIE tends to unity. Experimental data reveal that k_3 (186 ± 3 s^{-1} and 10 ± 0.1 s^{-1} for protium and deuterium, respectively) is essentially unchanged in MADH reactions performed in 30% glycerol. This kinetic behavior—i.e., essentially temperature-independent KIEs ($\Delta\Delta H^{\ddagger} = \Delta H^{\ddagger D} - \Delta H^{\ddagger H} = 1.0 \pm 2.9$ kJ mol^{-1}), but temperature-dependent rates ($\Delta H^{\ddagger H} = 42.2 \pm 1.1$ kJ mol^{-1}; $\Delta H^{\ddagger D} = 43.2 \pm 1.8$ kJ mol^{-1})—mirrors closely that seen in the absence of glycerol, indicating that quantum transfer, but not nuclear reorganization, is rate-limiting. When quantum H-tunneling is shown to limit—as is the case for MADH—H-transfer is analogous to established theory that describes long-range electron-transfer reactions in protein molecules *(34)*. Under these conditions, there is rapid destruction of coherent oscillations of the hydrogen between the wells by coupling to other degrees of freedom. Fast destruction of coherence relative to the coherent oscillations leads to a well-defined transfer rate and the Golden Rule applies—i.e., the transfer rate is proportional to the tunneling probability following distortion of the protein scaffold. H-transfer in MADH can thus be treated in the nonadiabatic limit. H-tunneling in MADH is thus conceptually similar to vibrationally assisted protein-mediated electron-transfer reactions that have been described *(34)*.

CONCLUSION

Our data for MADH illustrate that extreme (i.e., ground state) quantum tunneling driven by a thermally fluctuating potential energy surface is the mechanism of proton abstraction from the iminoquinone intermediate formed in the reductive half-reaction. Extreme tunneling is an attractive mechanism for cleaving stable C−H bonds—large activation energies make this energetically unfavorable for classical, over-the-barrier mode of breakage—and may be a general strategy employed by enzymes for catalyzing these 'difficult' transformations.

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