Vibrationally enhanced tunneling as a mechanism for enzymatic hydrogen transfer

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ABSTRACT We present a theory of enzymatic hydrogen transfer in which hydrogen tunneling is mediated by thermal fluctuations of the enzyme's active site. These fluctuations greatly increase the tunneling rate by shortening the distance the hydrogen must tunnel. The average tunneling distance is shown to decrease when heavier isotopes are substituted for the hydrogen or when the temperature is increased, leading to kinetic isotope effects (KIEs)—defined as the factor by which the reaction slows down when isotopically substituted substrates are used—that need be no larger than KIEs for nontunneling mechanisms. Within this theory we derive a simple KIE expression for vibrationally enhanced ground state tunneling that is able to fit the data for the bovine serum amine oxidase (BSAO) system, correctly predicting the large temperature dependence of the KIEs. Because the KIEs in this theory can resemble those for nontunneling dynamics, distinguishing the two possibilities requires careful measurements over a range of temperatures, as has been done for BSAO.

INTRODUCTION

In 1989, Cha et al. (1) announced that hydrogen (We use hydrogen as a generic term for protons, hydrogens, and hydrides; most of the issues we discuss here are independent of which electronic species is involved.) is transferred by means of quantum tunneling in the yeast alcohol dehydrogenase (EC1.1.1.1) reaction. Their evidence consisted of anomalies in the relationship between the hydrogen/tritium and deuterium/tritium KIEs that could not be explained by classical kinetics. Later that year Grant and Klinman (2) found that the bovine serum amine oxidase (BSAO, EC1.4.3.6) system exhibited hydrogen tunneling, observing, among other things, KIEs whose size (a hydrogen/tritium KIE greater than 50 at 5°C) could not be accounted for by classical kinetics. These results are certainly exciting in terms of strategies for catalysis and enzyme structure/function relationships, and they come at a time when sophisticated calculational tools (3, 4) are shedding light on hydrogen tunneling in systems of small molecules (5–8).

Unfortunately, the sheer size and complexity of enzymes makes direct application of most tunneling rate theories completely impractical. Moreover, no quantitative interpretation of the KIE data referred to above has been successful until now. Our goal in this paper is to bridge the gap between the chemical physics of hydrogen tunneling and the molecular complexity of biochemical systems. We do this first by emphasizing the importance of vibrational coupling to the tunneling event, an issue which has not been properly addressed in previous KIE calculations, and then by incorporating such coupling into a semi-empirical model which correctly predicts the temperature dependence of the KIE's in one of the above systems.

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Quantum mechanics in biomolecular reactions

The dynamics of large molecules is usually studied using only classical mechanics, partly because quantum simulations are intractable, and partly because of the widespread assumption that quantum mechanics can usually be ignored when discussing the dynamics of biomolecular systems. The recent experiments cited above certainly contradict that assumption, and in fact other examples of quantum dynamics in biological systems have been around for many years. The example which is most relevant to our problem is electron transfer.

In electron transfer reactions, including the primary events of photosynthesis, it is well established that the quantum dynamics of the transferred electron is crucial for understanding reaction rates; i.e., the electron tunnels (9, 10). In some electron transfer processes, it is also likely that the quantum character of atomic motion at the donor and acceptor sites plays a role in determining temperature and driving force dependencies of reaction rates, even at room temperature (11–14). Atomic tunneling may also be important in the dynamics of reactions at very low temperatures, as in ligand binding to heme proteins (15). Aside from these examples, however, it is widely believed that the molecular dynamics relevant to the determination of reaction rates in proteins are overwhelmingly classical (16, 17).

Hydrogen tunneling versus classical motion

As quantum effects are more important for lighter particles, hydrogen is the next candidate for transfer by tunneling. In gas phase there is little doubt that hydrogen

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tunneling is significant in transfer reactions at room temperature (18, 19), and much work has been devoted to developing useful and reliable methods for calculating such quantum effects (3, 20, 21). In contrast, most discussions of condensed phase hydrogen transfer assume that the reaction proceeds by classical activation of the hydrogen over a barrier, often with some small corrections for tunneling (19). The idea that the hydrogen tunnels from its ground state after some thermal motion of the enzyme or solvent was originally proposed long ago (22, 23), but was met with strong resistance (19). Tunneling of hydrogen from its ground state in condensed phase reactions has been supported by theoretical calculations in the context of at least one previous model (24), but these calculations did not lead to any convincing link with experiments. Other attempts to calculate measurable quantities starting from this idea (23) lead to predictions which have been refuted by experiment (2, 16). Earlier (25), we showed that models for ground-state hydrogen tunneling which treat the thermal fluctuations more realistically give results that qualitatively agree with experiment. This conclusion was reached independently by other authors (26). In this paper, we show that among simple models, ground state tunneling models with fluctuations are the best candidates for many reactions. We proceed to introduce a "saddle-point" calculation leading to a simple formula; we find that this formula gives a very convincing fit to experimental data which has not been fit by any other model.

Discussions of enzymatic hydrogen transfer most often assume that the hydrogen's motion is (at least approximately) classical (16, 17, 27), with two experimental facts being used to justify this view.

(*a*) When deuterons or tritons are substituted for the hydrogen the rate of transfer is typically slower by a factor roughly consistent with a classical (nontunneling) theory. This ratio of rate constants, called a kinetic isotope effect (KIE), is not nearly as large as what might naively be expected for a tunneling process.

(*b*) Transfer rates and KIEs are temperature dependent, in agreement with the standard, nontunneling theory. In contrast, static tunneling models suggest that rates and KIEs should be independent of temperature (23).

The experimental findings cited above, which seem to imply hydrogen tunneling, have motivated us to study several simple dynamical models for enzymatic hydrogen transfer in the hope of understanding the relative importance of classical and quantum transfer processes.

Before attempting to model any data, we will emphasize two main points which we believe are crucial to any discussion of enzymatic hydrogen transfer. The first is that comparison of quantum vibrational energy scales for the transferred hydrogen with typical classical energy scales, such as the activation energy, indicates that the reactive dynamics cannot be "classical" in any rigorous sense. The second is that thermal fluctuations in the structure of the enzyme active site have an enormous effect on the probability of tunneling, qualitatively changing the predicted rates, temperature dependencies, and isotope effects. While thermal motions have been included in previous models for hydrogen tunneling (24, 22, 23), these models missed the significance of protein motions for KIE experiments. We find that when the softness of the protein is taken into account by averaging over fluctuations of the size known to occur in proteins (28, 29), observations *a* and *b* can easily be accounted for. Moreover, these ideas lead to a quantitative theory of "vibrationally enhanced tunneling" from which we can derive a simple KIE expression capable of fitting the BSAO data in detail.

Vibrational "enhancement" versus "assistance"

In the case of electron transfer, the term "vibrationally assisted tunneling" usually refers to vibrations assisting the tunneling by modulating the relative energies of the initial and final state, allowing the tunneling event to occur when the states are of equal energy. As we discuss below, this same phenomenon is likely to take place in hydrogen transfer reactions, but our focus will be on another sort of vibrational coupling in which the vibrations enhance the tunneling rate by making the tunneling distance shorter (and/or the barrier height lower), thereby increasing the tunneling probability. This "vibrational enhancement" has been discussed in the context of electron transfer (30), but it plays a much more important role in the case of hydrogen transfer due to the hydrogen's larger mass and hence shorter penetration length. In particular, "vibrational enhancement" plays a crucial role in determining KIE's because the penetration length is isotope dependent.

"Classical" theory of KIEs

The theory which is generally used as the starting point for understanding KIE experiments asserts that the KIE arises from the larger quantum zero-point energy of the lighter isotope (27). This zero-point energy reduces the amount of thermal energy required to reach the top of the barrier, making the reaction more probable. Taking m_1 and m_0 to be the masses of the two isotopes, and k_{m_1} and k_{m_0} the respective reaction rates, this argument predicts

$$\ln \mathrm{KIE} \equiv \ln (k_{m_1}/k_{m_0}) = \left(\frac{1}{2}\hbar\omega_1 - \frac{1}{2}\hbar\omega_0\right)\frac{1}{k_B T} = \left(\sqrt{\frac{m_0}{m_1}} - 1\right)\frac{\hbar\omega_0}{2k_B T}, \quad (1)$$

where the ω's are the frequencies of the modes lost in the transition states, and k_B is Boltzmann's constant. This expression has three significant consequences. First, the KIE exhibits Arrhenius temperature dependence, and

should extrapolate to zero at infinite temperature. Second, the approximate magnitudes of the KIEs are determined, since the frequency of a C–H stretch is always ~3,000 cm^{-1}. The resulting H/T (hydrogen/tritium) and D/T (deuterium/tritium) KIEs should be ~22 and 2.6 (ln KIE = 3.1 and .94) respectively, at room temperature. Isotope effects smaller than these values are interpreted in terms of motion of heavier atoms along the reaction coordinate in the transition state (31), whereas larger isotope effects are conventionally taken as evidence for tunneling, at which point "tunneling corrections" are applied (19). Third, by taking the ratio of the logarithms of the H/T and D/T KIEs, the factor $\frac{1}{2}\hbar\omega_0/k_BT$ cancels, leaving a pure number,

$$\frac{\ln (\text{H/T KIE})}{\ln (\text{D/T KIE})} = \frac{\sqrt{3}-1}{\sqrt{1.5}-1} = 3.26, \qquad (2)$$

known as the Schaad-Swain exponent (32).

Note that while this theory treats the vibrational ground states quantum-mechanically, it treats motion across the top of the barrier classically. We will therefore refer to this as the Classical theory. We reserve the term "semiclassical" for the large body of methods, such as the WKB approximation, that are used to solve quantum problems in instances where Planck's constant may be regarded as small but nonzero. Purely quantum effects such as tunneling can be (and often are) calculated in a semiclassical approximation.

Can enzymatic hydrogen transfer be truly "classical"?

Classical mechanics is a good approximation when the spacings between energy levels (vibrational quanta) are much smaller than all characteristic classical energies in the problem. In the BSAO hydrogen transfer, the relevant vibrational quantum is governed by the C–H stretching frequency, $\omega/2\pi c \approx 3{,}000\ cm^{-1}$, so that $\hbar\omega \sim 36$ kJ/mol. The observed activation energy, which in the classical scenario sets an upper bound on the height of the barrier above the ground state, is only 56 kJ/mol (2). If the assumption of little or no tunneling were correct, there would thus be only one excited state below the barrier. In such a situation the existing classical models and tunnel correction schemes cannot be justified. The only way to escape this conclusion is to suppose that the potential surface becomes extraordinarily flat near the top of the barrier, so as to provide a vast increase in the density of states near the transition state. Such a disparity between the length scales for barrier width and binding seems ad hoc at best, especially since the proposed barrier height is much lower than the hydrogen binding energy. Simply put, vibration quanta are comparable to the barrier height, and therefore the hydrogen's motion is not classical.

The quantum approach

The natural quantum approach to this problem (which will give results equivalent to the classical model when the classical model is correct) is to think of the observed reaction rate as a Boltzmann-weighted average of the rate from each initial quantum state of the hydrogen. Because we have shown that there are probably only two or three initial states of motion along the C–H stretch that can contribute, this quantum approach seems appropriate. Even if excited states of the C–H bending modes of the transferred hydrogen are included, the number of terms in the average would be manageable. Unfortunately, calculating rate constants is always very difficult, and in many enzyme systems, including the BSAO system, lack of knowledge of the reaction site geometry renders rate calculations hopeless. We therefore focus our attention on calculating KIE's. The regime in which we carry out our calculations is one in which the ground state of the C–H bond makes the dominant contribution to the Boltzmann rate average. We will show that this regime is likely to apply to enzymatic hydrogen transfers at biological temperatures and below. The activation energy of the reaction comes from other degrees of freedom which are thermally activated, namely, low frequency vibrational modes whose dynamics may be regarded as independent of isotope and which enhance the tunneling rate. This picture is in sharp contrast to the classical theory, which supposes that only the hydrogen states above the classical barrier contribute to the rate, and to tunnel correction theories, which assume that states below the classical barrier make only a small contribution to the rate. It is interesting that attempts to use Bell's tunnel correction formulas frequently lead to a "tunnel correction factor" much larger than 1 (in reference 19, page 137, more than half the values given are greater than 2, the average value is greater than 5, and the largest value is 22, all at room temperature). In such cases Bell's formalism is not valid, and a theory which treats ground state tunneling correctly is more likely to be of use.

Tunneling through a static barrier

To illustrate the effects fluctuations have on tunneling kinetics, we will first consider a pure tunneling mechanism in the absence of fluctuations.

Tunneling is the process by which quantum particles penetrate classically forbidden regions. The probability of penetrating a barrier is given, in the WKB approximation (3) (with corrections smaller by a factor $\hbar/S$), by

$$P_{\text{tunnel}} \propto e^{-2S/\hbar}, \qquad (3)$$

where S, the WKB action, is defined by

$$S \equiv \int_a^b \sqrt{2m[V(x) - E]}\, dx. \qquad (4)$$

Here $V(x)$ is the potential energy barrier between reactant and product states of the hydrogen, and the limits of

integration are the turning points at the entrance and exit of the barrier where the potential energy equals the energy of the incident particle: $V(a) = V(b) = E$. Eqs. 3 and 4 imply that the tunneling probability decays exponentially with a (spatially varying) penetration length of $\hbar/2\sqrt{2m(V-E)}$. This barrier penetration probability is associated with a coupling or matrix element Δ between the initial and final states on either side of the barrier given in the WKB approximation by,

$$\Delta = \Delta_0 e^{-S/\hbar}. \tag{5}$$

The action S is the same as before, and Δ_0 can also be calculated.

In the simplest cases (e.g., Fermi's Golden Rule [29]) the rate for a reaction which proceeds by tunneling will just be proportional to the barrier penetration probability, i.e.,

$$k \propto \Delta^2 = \Delta_0^2 e^{-2S/\hbar}. \tag{6}$$

It is of obvious interest to compare the tunneling rate from the ground state with the rate of thermal activation over the barrier (the "classical" rate). One crude method of doing this is to compare the barrier penetration probability, $e^{-2S/\hbar}$, with the Boltzmann probability for reaching the top of the barrier, e^{-V/k_BT}. At sufficiently high temperatures, thermal activation will always dominate, whereas below a critical temperature, $T_c \approx V\hbar/2k_BS$, ground state tunneling will begin to dominate (33). To get a feel for this T_c, let us consider a "square" barrier of height 50 kJ/mol and width 1 Å. For this example, T_c turns out to be roughly 190 K. Different barrier shapes could give transition temperatures as much as a factor of two higher, whereas wider barriers would give a lower T_c. Thus, from a theoretical point of view that considers only static barriers, it is quite plausible that T_c is less than 300 K, implying no tunneling at or above room temperature.

From the experimental point of view, isotope effect measurements rule out ground state tunneling through static barriers: Eq. 6 implies a KIE of

$$\ln \text{KIE} = -(2S_1/\hbar - 2S_0/\hbar) = \left(1 - \sqrt{\frac{m_1}{m_0}}\right)2S_0/\hbar, \tag{7}$$

where S_0 denotes S calculated for a mass of m_0. This is a potentially huge isotope effect. For the square barrier discussed above, $S_0/\hbar$ (for tritium) would be $\sim$28. This would produce an H/T KIE of 10^{10}, whereas room temperature isotope effects larger than 30 are seldom seen. Only by assuming very short transfer distances can reasonable isotope effects be obtained, and it can be argued that the distances required are so short as to be energetically forbidden (19). Moreover, the KIE in Eq. 7 is independent of temperature, which also contradicts experiment.

Tunneling through a fluctuating barrier

When fluctuations are taken into account, the theory of hydrogen transfer by tunneling changes dramatically. As the protein undergoes low frequency, "breathing" vibrations, the active site is distorted so that the distance the hydrogen must tunnel changes. When the distance is longer, S is larger, so nothing happens. When the distance is shorter, however, S becomes smaller and tunneling becomes much more probable. Hence, *the reaction is controlled by the fluctuations to shorter transfer distances.* To illustrate the qualitative way in which fluctuations influence KIEs for tunneling, we begin with the simplest possible model that can incorporate what we believe to be the essential features of the problem. After discussing the qualitative implications of such a model, we present a generalization which is of significant quantitative use.

The rectangular barrier case

The thermal fluctuations of the protein produce a thermal distribution of the transfer distance, l. The fluctuations could represent the dynamics of a variety of vibrational degrees of freedom in both protein and substrate, as long as they are of low enough frequency to be treated classically. In this simplified model we take S to be proportional to l, i.e.,[1]

$$S \equiv \int_a^b \sqrt{2mV(x)}\,dx = \sqrt{2mV_{\text{eff}}}\,l. \tag{8}$$

This expression is correct for a rectangular barrier of height V_{eff}, and will actually allow us to calculate correct KIEs for a large class of potential surfaces, as discussed below. Our other temporary simplification is that we treat the protein's influence on l as that of a harmonic spring, which means the energy associated with deforming the protein to achieve some value of l is given by $U = \frac{1}{2}\kappa(l - l_{eq})^2$, where l_{eq} is the equilibrium value of l, and κ is the stiffness which resists changes in l. It is essential that U, the energy the system has when the transfer distance is l, not be confused with $V(x)$, the additional energy the system would have if the hydrogen were at the position x. In fact, the hydrogen's motion must be treated quantum mechanically, and the hydrogen can tunnel through the barrier $V(x)$ without changing the energy of the system at all. For the purposes of ground state tunneling, $V(x)$ is relevant only in that it defines the barrier shape for which S is to be calculated (Eq. 4) for each l, while $U(l)$ defines the energy needed to obtain a given l.

[1] We have omitted the E from Eq. 4. This is justified (assuming $V(x)$ is always measured relative to the bottom of the initial well) for tunneling from the lowest states since their energy is proportional to $\hbar$ which is assumed small in WKB. Rewriting the tunneling matrix element with S of this form can be done rigorously, resulting in a Δ_0 that is somewhat complicated, but having a known mass dependence of $m^{-1/4}$ (34, 35).

We will assume that at each l there is a well-defined tunneling rate proportional to the barrier penetration probability, and that the overall rate constant can be obtained by averaging this tunneling rate over the thermal distribution of protein configurations (values of l). This averaging procedure has been shown to be a useful way of calculating rates (36) and is justified whenever the dynamics of l can be described classically (37), provided the Boltzmann distribution is not significantly upset by the reaction dynamics, as we discuss below. (See "Some further questions" below.) The overall rate for ground state tunneling, then, is

$$k \propto \Delta_0^2 \int_0^\infty dl \exp[-\tfrac{1}{2}\kappa(l - l_{eq})^2/k_BT - 2\sqrt{2mV_{eff}}\,l/\hbar]. \quad (9)$$

Here, as stated earlier, we include only the contribution from the ground state of the C–H stretch. One could add terms to include excited states labeled by positive integers n, where each term would by multiplied by a factor $e^{-n\hbar w/k_BT}$ and would have V_{eff} replaced by $V_{eff} - n\hbar\omega$. As we shall discuss below, it is quite reasonable to believe that such terms will make only a small contribution to the rate at biological temperatures.

The integrand of Eq. 9 is a Gaussian curve whose peak (or "saddle" point) has been displaced along l to the position l_s. The value of l_s can easily be found to be

$$l_s = l_{eq} - 2\sqrt{2mV_{eff}}\,k_BT/\hbar\kappa. \quad (10)$$

Loosely speaking, l_s is the transition state for the protein coordinates: the reaction occurs when a fluctuation of the protein structure reduces l from l_{eq} to l_s, at which point the hydrogen tunnels. The value of l_s is determined by a compromise between the hydrogen not wanting to tunnel too far and the protein not wanting to distort too much; hence, l_s depends on mass and temperature. At higher temperatures, larger fluctuations are more important, and for larger masses, tunneling is dominated by shorter distances.

As long as l_s is not too close to zero ($l_s \gg \sqrt{k_BT/\kappa}$) we can safely extend the lower limit of the integral to $-\infty$ to obtain

$$k \propto \Delta_0^2 \exp\left(-2\sqrt{2mV_{eff}}\,l_{eq}/\hbar + \frac{4mV_{eff}k_BT}{\hbar^2\kappa}\right). \quad (11)$$

The reaction rate increases exponentially (although not in an Arrhenius manner) with temperature, even though the hydrogen is tunneling from its ground state, and the overall rate for tunneling is larger by an exponential factor than what one would calculate ignoring fluctuations. This rate enhancement factor can be written in terms of a more intuitive quantity—namely, the average (root-mean-square) fluctuation of the distance l, which we denote $\sigma = (k_BT/\kappa)^{1/2}$. In terms of σ, fluctuations increase the rate by a factor

$$\frac{k(\sigma)}{k(\sigma = 0)} = \exp(4mV_{eff}\sigma^2/\hbar^2). \quad (12)$$

With typical barrier heights $V_{eff} \approx 50$ kJ/mol, fluctuations on the scale $\sigma \sim 0.2$ Å, which are known to occur in the interior of proteins (28, 29), enhance the tunneling probability by a factor $\sim 10^9$ (!). This factor will make tunneling more important than the over-the-barrier mechanism at much higher temperatures than would otherwise be believable. The example which we earlier found to be dominated by tunneling below 190 K, now, in the presence of these fluctuations, has its transition temperature increased by a factor $1/(1 - 2mV_{eff}\sigma^2/S\hbar)$, to at least 550 K. (In fact, depending on how one extends the model to higher temperatures, one may find that at very high temperatures the reaction is dominated by fluctuations which destroy the barrier completely, so that over the barrier processes are not important at any temperature.) For many types of barriers, including the square barrier and any barrier which may be treated as parabolic above its ground state, $\partial^2 S/\partial E^2 \leq 0$ for all configurations of l. This can be shown to imply that all excited states will contribute less to the rate than the ground state when the temperature is below this fluctuation enhanced T_c (see Appendix B of reference 35). In this case, tunneling should dominate at any accessible temperature, and our treatment which neglects excited states of the C–H bond is justified. In general, one cannot be sure that no excited state makes a substantial contribution without investigating a detailed potential surface. For example, one could imagine that excited states of the C–H bending modes might contribute when the reaction is significantly noncollinear. In the limit that these modes are fully classical or fully quantum in character, they do not interfere with our analysis; in the quantum limit the modes tunnel from their ground state and can be included in S, whereas in the classical limit they can be included in U. Unfortunately neither limit may hold, and a rigorous treatment of such modes would complicate our equations immensely. Neglecting the effect of these modes, while not always ideal, is closer to the truth than summing over nonexistent states of arbitrarily low energy, as one must do implicitly when calculating large tunnel correction factors using the standard formulas. Thus, we include only the initial ground state, as is justified for a wide range of potential surfaces.

Returning to Eq. 11, we obtain the kinetic isotope effect

$$\ln \text{KIE} \approx \left(1 - \sqrt{\frac{m_1}{m_0}}\right) 2S_0/\hbar - (m_0 - m_1)\frac{4V_{eff}k_BT}{\hbar^2\kappa}, \quad (13)$$

where $S_0 = \sqrt{2m_0V_{eff}}\,l_{eq}$, which is the action for the reference particle (of mass m_0) to tunnel in the equilibrium geometry. This expression is temperature dependent and smaller in magnitude than that predicted for tunneling

through a static barrier. In terms of the parameters cited above,

$$\ln \mathrm{KIE} \approx \left(1 - \sqrt{\frac{m_1}{m_0}}\right) 2S_0/\hbar - 20\left(\frac{m_0}{m_p} - \frac{m_1}{m_p}\right)\left(\frac{\sigma}{0.2\ \text{Å}}\right)^2 \frac{V_{\text{eff}}}{50\ \text{kJ/mol}}, \quad (14)$$

where m_p is a proton mass. We see that the second term reduces the KIEs by an enormous factor relative to the predictions for tunneling through a static barrier. By varying the two free parameters, S_0 and $V_{\text{eff}}\sigma^2$, one can get any desired value for the KIE and its Arrhenius slope, and values in the range commonly assumed to be classical can be obtained with physically realistic parameters. This means that reactions thought to be classical based on the measurement of temperature dependent KIEs are also consistent with this scenario, in which the reaction proceeds purely by hydrogen tunneling. Clearly, the effects of fluctuations cannot be ignored.

The general case

Eq. 13 can qualitatively describe the BSAO data, and the fit can be improved by incorporating the logarithmic "prefactor" term discussed below. Quantitatively, however, the fit still leaves room for improvement (i.e., χ^2 is six standard deviations too big). We therefore, make a natural generalization of Eq. 9 which enables us to produce a quantitatively convincing fit to the data.

The rectangular-barrier model above assumes that the action S depends linearly on l and that the energy associated with distorting the protein, which we will refer to as U, is quadratic in l. For many different models it turns out that the average rate analogous to Eq. 11 is dominated by a small region of configurations l near l_s, so that only the behavior of U and S near l_s is relevant. This implies that the results of our rectangular barrier calculation will be correct for some large set of models in which the barrier need not be at all square, so long as the barrier changes its shape as l changes in such a way that $S(l)$ can be approximated as linear near l_s. We can generalize this by expanding both S and U to second order in the neighborhood of l_s:

$$S(l \approx l_s) = S(l_s) + S'(l_s)(l - l_s) + \tfrac{1}{2}S''(l_s)(l - l_s)^2 + \cdots,$$
$$U(l \approx l_s) = U(l_s) + U'(l_s)(l - l_s) + \tfrac{1}{2}U''(l_s)(l - l_s)^2 + \cdots. \quad (15)$$

This expansion allows an accurate evaluation of the thermally averaged tunneling rate for a wide range of functions S and U; in particular, U need not be a harmonic function of l. Furthermore, although it is convenient to think of l as the width of the barrier, this need not be the case. To consider an unphysical example, imagine a system in which the width of the barrier remained absolutely fixed, but the height fluctuated. Then, l would represent the displacement of whatever coordinate was responsible for the height fluctuations, and have nothing to do with the barrier width, but the various derivatives of S and U with respect to l would still make sense. Thus, in using Eq. 15 we make no assumption about what combination of height and width fluctuation is occurring. Indeed, l can also consist of more than one coordinate (35), and the tunneling trajectory (denoted by x in Eq. 4) can include bending as well as stretching movements of the hydrogen.

As before (Eq. 9), the thermally averaged rate constant is calculated by integrating over fluctuations in l:

$$k \propto \Delta_0^2 \int_{-\infty}^{+\infty} \exp[-2S(l)/\hbar - \beta U(l)]\, dl, \quad (16)$$

where β is $1/k_B T$. The validity of the expansion in Eq. 15 for evaluating Eq. 16 relies on the temperature being low compared to features in the protein's energy surface, U, and on $\hbar$ being small relative to S. In fact, we have studied some more complex model surfaces and find that, in reasonable parameter regimes, contributions from higher derivatives (S'''', U'''', . . .) are negligible if one studies KIEs over a limited range of temperatures (0 to 45°C), as in most experiments on enzymes.

The integral in Eq. 9 is still Gaussian and yields

$$\ln k \propto -2S - \beta U + \frac{(2S' + \beta U')^2}{2(2S'' + \beta U'')} + 2\ln(\Delta_0) - \tfrac{1}{2}\ln(2S'' + \beta U''), \quad (17)$$

where the variables U, S, and their derivatives are all to be evaluated at the dominant configuration, l_s, which is determined by

$$(2S' + \beta_0 U')|_{l=l_s} = 0, \quad (18)$$

β_0 being the reciprocal of some reference temperature (.0035 K^{-1} for our purposes). To simplify notation we have absorbed the $1/\hbar$ into S and its derivatives.

To convert Eq. 17 into an isotope effect, we use the fact that S is proportional to $\sqrt{m}$, and that Δ_0 is proportional to $m^{-1/4}$ (34, 35); we ignore other logarithmically small corrections which might arise due to the possibility of Δ_0 depending on l (and therefore on m). The result is

$$\ln(\mathrm{KIE}) = (1 - \sqrt{m})2S + 2S^2\left[\frac{(\sqrt{m} - \beta/\beta_0)^2}{\sqrt{m}2S'' + \beta U''} - \frac{(\beta/\beta_0 - 1)^2}{2S'' + \beta U''}\right] + \tfrac{1}{2}\ln\left(\frac{2S'' + \beta U''}{\sqrt{m}2S'' + \beta U''}\right) - \tfrac{1}{2}\ln m, \quad (19)$$

where m denotes the ratio of the two masses, so that $\mathrm{KIE} \equiv k(m_1)/k(m_0)$, and $m \equiv m_1/m_0$. As the experiments on BSAO measure the H/T and D/T KIEs, it is convenient to choose m_0 to be the mass of tritium, so that only m will be different for the two experiments.

The limit of Eq. 19 when $\beta U'' \gg 2S''$ leads to

$$\ln(\mathrm{KIE}) = \tfrac{1}{2}\ln(m) + (1-\sqrt{m})\left(2S + \frac{4S'^2}{\beta_0 U''}\right) - (1-m)\frac{2S'^2}{\beta U''}, \quad (20)$$

which is the same as the "simple model" result we had earlier, except that we now include the logarithmic term that comes from Δ_0. In the opposite limit, $2S'' \gg \beta U''$, we obtain

$$\ln(\mathrm{KIE}) = -\frac{3}{4}\ln(m) + (1-\sqrt{m})\left(2S - \frac{S'^2}{S''}\right) + \left(\frac{1}{\sqrt{m}} - 1\right)\beta^2\frac{S'^2}{S''}, \quad (21)$$

This limit is actually more natural, in that U involves distorting the protein and therefore might be expected to vary over longer length scales than S, which involves moving just the hydrogen. In this limit, the theory has only two adjustable parameters, S and S'^2/S''.

Comparison with experiment

The two parameter theory described here can account for the four experimentally known quantities: the H/T and D/T isotope effects, and their temperature dependencies. As noted above, Klinman and co-workers have made such measurements on yeast alcohol dehydrogenase (YADH) and on bovine serum amine oxidase (BSAO). In both cases, considerable care was taken to insure that the measured isotope effects were not significantly contaminated by the complexity of the reaction sequence, and in both cases interesting departures from classical expectations were observed. We focus on BSAO because in this enzyme, with benzylamine as a substrate, secondary isotope effects are relatively small, simplifying the theory (see below).

In BSAO the absolute magnitude of the isotope effects is larger than one expects classically, suggesting that tunneling is important. There are also large differences in activation energy among the different isotopes, which is inconsistent with ground state tunneling through a static barrier, and could not be accounted for within the usual models for "tunneling corrections" (2). Also, the Schaad-Swain relation (Eq. 2) holds for this system (2), contrary to what the tunnel correction theory predicts.

In Fig. 1 we show the data of reference 2 and the curves generated using a χ^2 fit of the two free parameters in Eq. 21. The quality of the fit is statistically indistinguishable from a four parameter (two slopes, two intercepts) Arrhenius fit. In fact, χ^2 is slightly better for our two parameter fit than for the four parameter fit, which means that the slight curvature that our model predicts is reflected in the data, but not at a statistically significant level.

As Eq. 21 implies that an Arrhenius plot of the KIE should show a specific amount of curvature, it is of obvious interest to discuss whether this curvature can be confirmed experimentally. If the one-sigma error bars on H/T KIE in BSAO were reduced to one per cent over the existing range of temperatures, the curvature of ln (KIE) in Eq. 21 would be distinguishable from a pure Arrhenius relation by two standard deviations. Such small error bars are not unrealistic (in fact, they have been achieved in some of the existing data). Unfortunately, Eq. 21 is not the only way of fitting the existing data; good fits can be achieved using Eq. 19 with values of U''/S'' anywhere from 0 to 0.35. The curvature of ln (KIE) at $U''/S'' = 0.35$ is only one-tenth of what it is in Eq. 21, and therefore can only be detected with error bars of 0.1%. While 0.1% may be unattainable, any improvement of the error bars would put stronger constraints on U''/S'', and it may well be that the true value of U''/S'' would imply a measurable curvature.

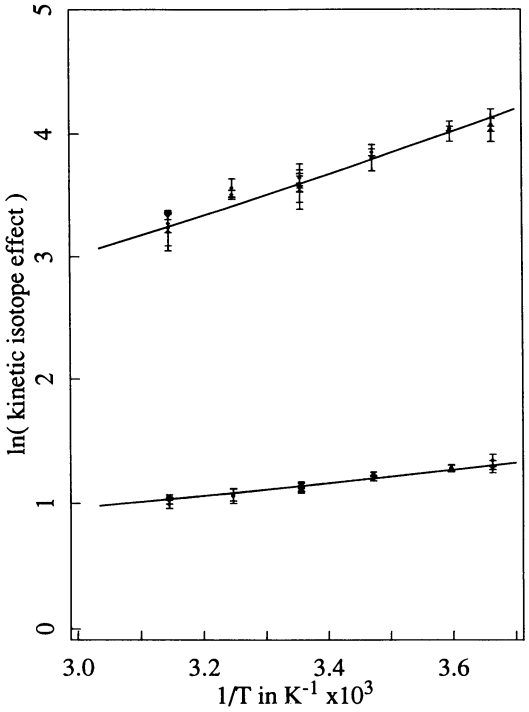

FIGURE 1 Grant and Klinman's experimental data on the BSAO system (18), plotted with our fit from Eq. 21. The upper set of data are the H to T rate ratios at different temperatures; the lower set are the D to T ratios. The two curves were generated using the same values of the free parameters, S_0 and S'^2/S''. The values are given in Eq. 22. Although the lines appear straight, they are actually parabolas centered at $1/T = 0$.

The parameters we obtained by fitting the data to Eq. 21 are,

$$\frac{S_0}{\hbar} = 2.01 \pm .02 \approx 2 \quad \text{and} \quad \frac{S_0'^2}{\hbar S_0''} = 4.24 \pm .07 \approx 4. \quad (22)$$

These were calculated at a reference temperature of $T_0 = (1/.0035)\mathrm{K} = 286\mathrm{K}$, and reflect values at the dominant configuration, l_s, for the reference mass, m_0, which is tritium. Error bars in the parameters are just experimen-

tal errors propagated through the fitting procedure, and therefore represent how much the parameters can be changed before the quality of the fit deteriorates; theoretical uncertainties (due to higher derivatives of U or S, or due to break-down of the WKB approximation, for example), are not included.

To get a feeling for what these values mean, we have calculated the WKB action for two superposed Morse potentials as a function of their separation, l. Morse potentials have two parameters, which we take as the C–H bond energy, $D \approx 4$ eV and the vibrational frequency, $\omega_H/2\pi c \approx 3{,}000$ cm^{-1}. Then to match the value of S_0, the transfer distance for hydrogen in the dominant configuration would be $l_s \approx 0.9$ Å, while for tritium $l_s \approx 0.8$ Å. We see that l_s is smaller for tritium, as it must be. Both values seem plausible and do not violate energetic considerations of reference 19 (pages 183–184). The value of l_{eq} is known to be approximately 1.5 Å in the YADH system (1), but is not known for BSAO. These values of l_s can be used to estimate σ, the root-mean-squared thermal fluctuation in l, since we know the temperature and activation energies. An approximate relation is that $\sigma^2 \approx (l_{eq} - l_s)\delta l_s k_B T/\delta E_a$, where E_a is an activation energy, and δ means the change from one isotope to another. We find that σ is in the range of .1–.15 Å, which is consistent with what is known about protein motions (28, 29).

The Morse potentials are not intended as a realistic potential surface. Indeed, differentiating the action with respect to the separation of the two minima leads to a value for $S'^2/\hbar S''$ of ~20 instead of 4. In trying to find a simple potential surface which could incorporate both of our free parameters, we noticed that in Eq. 22, $S'^2/S'' \approx 2S$. This relation will be satisfied whenever $S \propto l^2$. For a square barrier of fixed height, we know $S \propto l$, but for any barrier whose height scales as its width squared, $S \propto l^2$ will hold. Such is the case for a parabolic barrier whose imaginary frequency is held fixed as its width fluctuates, or for a quartic double well,

$$V(x) = -\tfrac{1}{2}m\omega^{\ddagger 2}(x^2 - 2x^4/l^2). \qquad (23)$$

This potential will achieve the values found in Eq. 22 provided $\omega_H^{\ddagger} l_{sH}^2 = 500$ cm^{-1} Å^2. Choosing $\omega^{\ddagger}$ to be ~1,200 cm^{-1} as calculated for the YADH reaction (38), we find a hydrogen transfer distance $l_s \approx .6$ Å, and .4 Å for tritium, in the dominant configuration. These distances are large enough to be consistent with Bell's energetic analysis ([19], pages 183–184), and give a value of σ in the same range as for the Morse potentials. Still, a quartic is a highly constrained form for a potential surface and is unlikely to reproduce the true surface in any detail. Our point is that the parameters arrived at in Eq. 22 represent sensible values.

Another interesting consequence of the observation that $S'^2/S'' = 2S$ is that it makes the $(1 - \sqrt{m})$ term in Eq. 21 vanish. Neglecting the small, logarithmic term, Eq. 21 is reduced to a single term whose mass dependence is identical to the classical KIE expression (Eq. 1), which means that the classical Schaad-Swain relationship (Eq. 2) is satisfied. While deviations from the Schaad-Swain relationship are often sought as evidence for nonclassical behavior, we see here that it is not hard for the relationship to be satisfied in reactions that proceed by vibrationally enhanced tunneling. The classical and tunneling scenarios can be distinguished even when the Schaad-Swain relationship holds, however. In the classical case, the logarithm of the KIE should extrapolate to zero at infinite temperature on an Arrhenius plot, whereas, the β^2 temperature dependence of Eq. 21 would have twice this much Arrhenius slope, as is approximately the case for the BSAO data.

Some further questions

We now address in a preliminary way some of the qualitative questions which arise when thinking about the dynamics of hydrogen transfer via vibrationally enhanced tunneling.

Entropic effects

One assumption that we made in our calculation is that the statistics of the l coordinate are determined by an energy surface $U(l)$. To be more general, we should interpret U as the free energy, to allow for changes in entropy that accompany motion along l. Thus, $U(l)$, the free energy, is really a function of both l and the temperature, and our above calculation of the temperature dependence of the KIEs is not fully general.

Such effects will not be significant when the dominant forces near l_s are due to direct electrostatic interaction of the donor and acceptor sites (see reference 19), since changes in entropy that do not occur near l_s will cancel out in KIE measurements. On the other hand, it is not difficult to generalize our model if one is willing to pay the price of additional free parameters.

For example, if the derivative of the entropy, $\mathcal{S}$ (not to be confused with the action S), is roughly constant near l_s, one finds that the temperature dependent part of Eq. 21 is altered:

$$(\beta S')^2 \rightarrow (\beta S' + \tfrac{1}{2}(\beta_0 - \beta)\mathcal{S}')^2. \qquad (24)$$

Note that if experiments are done at only one temperature then $\beta = \beta_0$ and there is no effect; i.e., the Arrhenius slope of the KIE is affected, but the KIE itself is not. (That is, the original KIE expression, in terms of quantities measured at l_s defined at some reference temperature, is correct. The proper determination of l_s, however, should be made taking U to be the free energy, which depends on entropy. Thus, changing the gradient of the entropy will change the magnitude of the KIE, but not the expression for it.) The temperature dependence will still be a parabola, although its center will be moved from $\beta = 0$ to $\beta = \mathcal{S}'/(2S' - \mathcal{S}')$. Of course, more com-

plicated equations will result if higher derivatives of the entropy are included. Including further terms in the free energy, such as the heat capacity, can alter the curvature and produce cubic and higher order terms in the Arrhenius plot, but will not disturb the expressions for the KIE or its Arrhenius slope near any given reference temperature.

We conclude here that since we can fit the BSAO data without considering entropic corrections, $\mathcal{S}'$ must be small near l_s. This means only that entropic forces are small near the dominant tunneling configuration, and does not imply that the entropic contribution to the free energy of activation is small. So long as $\mathcal{S}'$ is small, $\mathcal{S}(l_0)$ can differ from $\mathcal{S}(l_s)$; and only overall rates, not KIEs will, be affected.

Driving force effects and the requirement for degeneracy

Tunneling is significant only between states of nearly equal energy. What happens if the reactant and product states have different energies, that is, if there is a nonzero thermodynamic driving force for the reaction? As in the case of electron transfer, we expect there to be motions of the protein which modulate the energy difference between reactants and products, so that if the two states are not in degeneracy to begin with, the tunneling event waits until some dynamic fluctuation brings them near degeneracy. In fact, this is what is normally meant by the term "vibrationally assisted tunneling" when it appears in the electron transfer literature. When the temperature is high compared to $\hbar$ times the frequency of the mode doing the assisting, one will observe a contribution to the activation energy for tunneling from the energy required to deform the molecule into a configuration having degenerate initial and final states (9, 39, 26). Since this component of the activation energy depends on the driving force for the reaction but not on the mass, the rate will exhibit an isotope effect independent of driving force. Alternatively, if the energy level fluctuations are too small to allow degeneracy, one should observe potentially large changes of KIE with driving force. As in the case of electron transfer (11), studies of driving force dependencies for rates and KIEs in a homologous series of substrates will be critical in deciding which dynamical picture is relevant.

Coherence

The possibility of tunneling between states localized on opposite sides of a barrier leads, by itself, only to coherent oscillations of the hydrogen back and forth between the two wells. Coupling to other degrees of freedom in the environment destroys this coherence and leads to a well-defined rate of transfer (40). We have assumed that this destruction of coherence is fast compared to the frequency of coherent tunneling oscillations, so that the coherent mixing cannot progress to any significant extent before it is interrupted. Under these conditions, the Golden Rule (41) is applicable, and there is a well-defined rate proportional to the tunneling probability at each protein configuration, l; this is similar to the nonadiabatic limit of electron transfer theory (9, 42). In the opposite limit, coherent mixing is so rapid (or the coherence time so long) that one should really treat the tunneling nonperturbatively, diagonalizing the Hamiltonian at each value of l. This is the analog of the Born-Oppenheimer approximation for molecular electronic structure and corresponds to the true adiabatic limit of electron transfer theory. We feel that this situation is unlikely in a protein environment, although essentially this approach has been taken for hydrogen transfer between small molecules (21).

"Adiabaticity"

Let us imagine that coherence is destroyed rapidly, resulting in a well-defined rate at each l, but that motions along l itself are very slow. In this limit the Boltzmann distribution of l is not maintained, and the rate at which the protein can diffuse into and out of the critical configurations near l_s will at least partly control the observed reaction rate. This is reminiscent of "adiabatic" electron transfer in polar solvents, where the observed rate is proportional to the dielectric relaxation rate, not the electron tunneling probability (43). ("Adiabatic" here means that the rate is independent of the electronic matrix element, not that one can calculate the rate using the Born-Oppenheimer approximation.) If we adopt a strict analogy with the electron transfer problem, the rate becomes independent of the tunneling probability and hence (approximately) independent of isotope, despite the fact that the reaction proceeds purely by tunneling! The rates will depend, however, on the time scales characterizing the motion along l, which with some luck will be affected by changes in the solution viscosity; this would cause viscosity dependent rates, as in Kramers' classic discussion (44). In fact, the analogy with electron transfer is incomplete, since the dominant configuration l_s is isotope dependent, leading to an interplay between the dynamics along l and the dynamics of the tunneling event itself. This suggests that not just the rates, but the isotope effects themselves are viscosity-dependent in this regime. This would provide an unambiguous signature of the interactions between protein motions and the dynamics of the hydrogen transfer event.

Secondary isotope effects

Another approach to the study of hydrogen transfer reactions is to examine the kinetic effects of isotopic substitutions at sites other than the transferred atom or ion. We have been able to give a relatively simple and general discussion precisely because we ignore such effects; it is only when a single particle tunnels that the tunneling probability depends on mass in a simple way. If the transfer is accompanied by significant displacements of a second hydrogen and the frequency of the secondary coordinate is comparable to the frequency of the primary, then there is no general formula for the dependence of

the tunneling probability on the mass of either hydrogen. Arguments based on "effective mass" or corrections to the activation energy based on changes in zero-point energy are only valid if there is a clear separation of vibrational frequencies (45). Obviously, if the secondary effects are substantial, the calculations presented here for the primary KIE will be incorrect; fortunately for BSAO the secondary KIEs are around 1.2, compared to ~50 for the primary. The situation is very different in the case of YADH (1), where an analysis of two dimensional tunneling seems essential. The theory will simplify once again if the second atom is always much heavier than the transferred atom, which suggests that ^{14}C isotope effects will be very informative, especially if coupled with spectroscopic studies of the relevant vibrational modes in the substrate.

Concluding remarks

In the last decade there has been an enormous increase in our knowledge about protein dynamics, and there have been persistent hints from the electron transfer literature that quantum effects can contribute to the control of biochemical reaction rates even at room temperature. Despite these advances, most current descriptions of enzyme mechanism involve relatively static, classical pictures. We have argued here that such pictures are wrong for most hydrogen transfer reactions. Quantum energy scales are comparable to observed activation energies, so that no truly classical approximation can be valid. Quantum tunneling is so sensitive to transfer distance that realistic structural fluctuations at the active site have an enormous qualitative effect on the transfer rate and on the relative importance of classical and quantum transfer mechanisms. These arguments lead to a family of models for "vibrationally enhanced tunneling," within which we have found a simple two-parameter description of recent isotope effect experiments on bovine serum amine oxidase.

Unlike BSAO, most enzyme-catalyzed reactions exhibit KIEs which fall within the range required by classical theories. Nonetheless, the comparison of vibrational quanta with observed activation energies suggests that even in these cases a classical description is unlikely to be theoretically consistent even if it does explain some aspects of the data. It is, thus, particularly interesting that vibrationally enhanced tunneling can produce tunneling KIEs in the same range as those expected for a classical over-the-barrier reaction.

We have shown that the usual reasons for rejecting tunneling as a significant factor in enzymatic hydrogen transfer are wrong. Whether vibrationally enhanced tunneling proves to be a generally applicable scenario for hydrogen transfer remains to be seen. Our KIE expression, though not incorporating all possible complications we discussed, appears to be a useful starting point for interpreting the KIE's. In particular it shows that the sizes of the KIEs are insufficient to distinguish tunneling from non-tunneling kinetics, and demonstrates the value of the temperature dependence of the KIEs in this regard.

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