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CHAPTER 17

INTERPRETATION OF KINETIC ISOTOPE EFFECTS IN ENZYMATIC CLEAVAGE OF CARBON-HYDROGEN BONDS

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Abstract: A model is presented which relates kinetic isotope effects and their temperature dependence to physical parameters governing enzymatic carbon–hydrogen cleavage, a reaction that typically exhibits a large isotope effect indicative of tunneling. The model aims to replace the Arrhenius equation, which is not valid for tunneling reactions, and the one-dimensional Bell model, which excludes skeletal vibrations. Cast in the form of three user-friendly analytical equations, it applies directly to the observed rate constants and effective activation energies, for both adiabatic and nonadiabatic hydrogen transfer. The approach has the dual aim of establishing criteria to probe whether a set of data can be assigned to a single rate-limiting tunneling step and to find numerical values for the proton or hydrogen transfer distance and for properties of the tunneling mode as well as skeletal modes involved in this step. It is applied to several enzymatic (model) reactions, including free-radical reactions catalyzed by cofactor B₁₂ and phenylalanine hydroxylase, and electron-assisted proton-transfer reactions catalyzed by soybean lipoxygenase-1 and methylamine dehydrogenase.

Keywords: Enzymatic reactions, proton tunneling, CH-bond cleavage, deuterium isotope effects

17.1. INTRODUCTION

In many enzymatic CH-cleavage reactions the CH bond is broken by transfer of the hydrogen to an acceptor, usually an oxygen or other carbon atom. If, upon replacement of hydrogen by deuterium (or tritium), the rate of such a reaction is strongly reduced, it is evident that transfer occurs by quantum-mechanical tunneling and that this tunneling is, by itself or in combination with other steps, rate-limiting. The

question to be addressed in this contribution is whether it is possible to go beyond these obvious conclusions and draw additional information from the magnitude of a kinetic isotope effect (KIE) and its temperature dependence. Elsewhere [1] we showed that there is no apparent common pattern among the various enzymatic CH-cleavage reactions for which data are available. Given the complexity of enzymatic processes, direct attempts to calculate these properties run into many problems. To be at least partly realistic, elaborate calculations are required that nevertheless cannot avoid arbitrary cut-offs. There are always too many variables and therefore it is unlikely that a particular fit yields unambiguous information on structure or mechanism. The problem is compounded if the data include several reactions involving minor variations in enzyme or substrate. Obviously, the successful reproduction of an observed KIE of a single enzyme-substrate combination at a single temperature will tell us more about the theoretical methods used than about the enzymatic system studied. Moreover, the implied assumption that the reported kinetic data pertain to a kinetically isolated single step in the enzymatic reaction sequence may not even be valid.

Experimentally, the hydrogen transfer rate constant is usually measured as the initial rate of product formation under excess substrate conditions. This follows from the Michaelis-Menten equation, which in its simplest form is given by

$$k_{t \rightarrow 0} = k_{\text{cat}}[\text{SH}] / ([\text{SH}] + K_{\text{M}}) \quad (17-1)$$

where $[\text{SH}]$ is the substrate concentration and $K_{\text{M}} = (k_{-1} + k_{\text{cat}}) / k_1$ is the Michaelis constant, k_1 and k_{-1} being the association and dissociation rate constants, respectively, of the reactive complex formed by enzyme and substrate, and k_{cat} is the hydrogen transfer rate constant.

For most chemical reactions the rate constant $k(T)$ depends exponentially on the temperature, as expressed by the Arrhenius equation

$$\ln k(T) = \ln A - E_a / k_B T \quad (17-2)$$

where E_a is the activation energy and $k_B T$ the thermal energy. Pictorially, it describes the reaction as a transition over a potential-energy barrier with height E_a , the frequency factor A being the rate at which the barrier is attacked by collisional, diffusional or vibrational motions. Evidently, this picture is not valid for light particles such as protons that need not go over the barrier but can tunnel through it. Nevertheless, the rate of tunneling reactions, in particular those observed in biological systems, is generally reported in terms of Arrhenius parameters. Although for such reactions these parameters are no longer constants, they appear to be constant if measured over the narrow temperature interval to which biological systems are usually constrained. They are also isotope-dependent, although the potential energy and thus the barrier is mass-independent. Obviously, they have lost their original physical meaning: E_a is no longer the barrier height, i.e., the energy of the transition state, and A is no longer the attack frequency characteristic of the reaction coordinate. Without further modeling of the tunneling process, these parameters are quite meaningless.

The fact that, depending on its energy, the tunneling particle may penetrate the barrier at different points and follow paths of different lengths undoubtedly complicates the picture. Moreover, the barrier itself is not static, as in the Bell model [2], but subject to the vibrations of the molecular skeleton. However, these complications are offset by the existence of different hydrogen isotopes; their different masses leave the barrier essentially untouched but greatly affect their ability to penetrate it. This allows us to analyze tunneling reactions to the same extent as classical reactions, although the methods are different and, admittedly, more complicated. However, the very complexity of enzymatic processes implies that even the most sophisticated methods currently in use can only deal with an isolated step in the reaction chain and can therefore produce only approximate answers. In this contribution, we take advantage of this situation by simplifying the treatment of the tunneling step to the point where it can be used routinely for the interpretation and presentation of tunneling data. In contrast to the Arrhenius method and its adaptations based on the Bell model, we start from premises that are fundamentally correct, even if the approximations introduced to achieve tractability will undoubtedly cause inaccuracies. However, these approximations are not fundamental to the method and can be gradually removed if more information becomes available and if the kinetic data warrant it. Our approach, even in its simplest form, will provide answers on the role of skeletal vibrations in the tunneling reaction, answers that cannot be obtained from the one-dimensional Arrhenius/Bell approach. This role is a fundamental aspect of the specificity and efficiency of enzymes.

The need to include skeletal modes explicitly in the modeling follows, firstly, from the fact that such modes can modulate the barrier width and thus the tunneling distance, and, secondly, from the fact that they, rather than the high-frequency CH- and CD-stretching vibrations of the tunneling coordinate, govern the temperature dependence of the tunneling process. This realization is far from new [3–8], but earlier implementations rely on elaborate calculations that are overly complex and thus unattractive, and/or parameters that are not easy to guess. The new aspect of our modeling is that it is analytical and leads to formulas that apply directly to the observed rate constants. A full description together with application to a specific set of kinetic data is published elsewhere [8]. The application is to reactions catalyzed by soybean lipoxygenase-1, studied by Klinman and coworkers [5, 6]; it includes not only an analysis of the rate constants and their temperature and isotope effects, but also an analysis of the effect of six mutations, leading to several new results. Questions addressed, apart from the basic question whether the data are representative of a single rate-limiting tunneling step, include the magnitude of the tunneling distance, the properties of the skeletal modes involved in the tunneling, and, in general, the mechanism of the tunneling reaction.

Two such mechanisms have been found to be operative for enzymatic CH cleavage. The first and presumably older involves a free radical that abstracts the hydrogen by valence forces. Such a mechanism is well suited to hydrogen transfer between two carbon centers since it is operative over large distances. However, its long range turns it into a ‘brute force’ mechanism that runs the risk of causing

collateral damage, a problem that is avoided by the evolution of a more gentle ionic mechanism involving an atom or group that withdraws an electron from the CH bond to initiate a three-center process in which this bond donates an electron to one receptor and a proton to another. This mechanism, usually referred to as electron-assisted or nonadiabatic proton transfer, uses van der Waals rather than valence forces and involves shorter transfer distances to a proton receptor such as an oxygen center that engages in hydrogen bonding. As we show in this contribution, the analysis we propose can determine, on the basis of the observed KIE and its temperature dependence, which mechanism prevails in a given enzymatic reaction.

In the next section we briefly review the model and quote the equations to be used in the analysis of the observed kinetic data. These equations together with standard parameter values are used to determine to what extent the tunneling step can be identified as rate-limiting. If the data pass this test, qualitative and quantitative information can be obtained on the mechanism of the tunneling reaction and the structure of the reaction center. In subsequent sections the model is applied to a representative set of kinetic data on enzymatic systems and models thereof, including soybean lipoxygenase-1 (SLO1) [5, 6], coenzyme B₁₂ [9, 10], primary amine dehydrogenases [11, 12], and phenylalanine hydroxylase [13].

17.2. MODEL

To replace the Arrhenius Eq. (17-2), which, as pointed out above, is not valid for tunneling reactions, we use an equation derived in [8] from Fermi's Golden Rule, which in its simplest form reads:

$$\ln k(T) \simeq \ln \left[\frac{2\pi}{\hbar} J^2 \varrho(E_f) \right] - \frac{r_0^2}{2a_0^2 + A^2(T)} - \Delta E^{\text{eff}}/k_B T \quad (17-3)$$

The derivation applies explicitly to tunneling, primarily to adiabatic transfer, but, as shown in [8], it can also be used for nonadiabatic reactions provided it is applied to relative rather than absolute rate constants. The form of the first term on the rhs follows immediately from the Golden Rule. J is an electronic coupling term and $\varrho(E_f)$ is the density of final states; both parameters are assumed to be isotope- and temperature-independent and will not concern us further. The second term represents the behavior of the vibrations directly involved in the transfer. The parameter r_0 denotes a particular value of the tunneling coordinate r , namely the equilibrium transfer distance, defined as the separation of the two equilibrium positions of the tunneling particle, H or D. The parameter a_0 (c.q. a_{eff}) represents the harmonic (c.q. anharmonic) zero-point amplitude of the XH-stretching vibration along the tunneling coordinate r , where X denotes C, O, or whatever atom carries the tunneling particle. If X in the final state differs from X in the initial state, we take an average value for a_{eff} . Thus for $A(T) = 0$, the second term represents the exponent of an overlap integral between zero-point vibrations in a static

double-well potential formed by two equivalent parabolas displaced by r_0 . If an excited vibrational level is involved, there will be additional terms, but these are nonexponential and tend to be unimportant for relative rate constants.

The term $A(T)$ represents the effect of those skeletal vibrations that ‘promote’ or ‘gate’ tunneling. These modes have components R parallel to the tunneling coordinate r and are symmetric relative to the dividing plane between the initial (reactant) and final (product) states. They effectively shorten the tunneling distance [3, 14]. Finally, ΔE^{eff} is a term that includes any endothermicity, presumed to be small in typical enzymes, as well as contributions from those skeletal modes that are antisymmetric relative to the dividing plane of the reaction; since they have the same symmetry as the tunneling mode, they can contribute to the barrier. Thus they hinder tunneling similar to the Marcus-type exponent describing polar-solvent reorganization. We will assume that the temperature- and isotope-dependence of this term is weak and can be neglected. As the notation emphasizes, we assume that only the skeletal amplitudes are temperature-dependent at biologically relevant temperatures. For a critical discussion of the various approximations used to derive Eq. (17-3) we refer to [8]. To relate the CH and CD modes, we set $m^{\text{D}} = 2m^{\text{H}} = 2$, so that $(a_{\text{eff}}^{\text{H}})^2 = \sqrt{2}(a_{\text{eff}}^{\text{D}})^2$.

Since many of the parameters in Eq. (17-3) are irrelevant to our purpose, we simplify the notation by keeping only those that are needed and expressing all lengths in units $a_{\text{eff}}^{\text{H}}$. The new parameters

$$L = \ln\left[\frac{2\pi}{h}J^2\varrho(E_f)\right]; \quad \rho = \frac{r_0^2}{2(a_{\text{eff}}^{\text{H}})^2}; \quad z(T) = \frac{A^2(T)}{2(a_{\text{eff}}^{\text{H}})^2}; \quad B(T) = \Delta E^{\text{eff}}/k_B T \quad (17-4)$$

reduce Eq. (17-3) to

$$\ln k^{\text{H}}(T) = L - \frac{\rho}{1 + z(T)} - B(T), \quad \ln k^{\text{D}}(T) = L - \frac{\rho\sqrt{2}}{1 + z(T)\sqrt{2}} - B(T) \quad (17-5)$$

In this notation $z(T)$ and $z(T)\sqrt{2}$ are the contributions of the skeletal modes to the transfer relative to the contribution of the H and D tunneling modes, respectively. This contribution is both isotope- and temperature-dependent and turns out to be a key parameter in the analysis. It enters the expression for the KIE, denoted by $\eta = k^{\text{H}}(T)/k^{\text{D}}(T)$ in the form

$$\ln \eta = \frac{\rho(\sqrt{2} - 1)}{[1 + z(T)][1 + z(T)\sqrt{2}]} \quad (17-6)$$

Hence within the model the KIE is fully determined by the transfer distance, represented by ρ , and the contributions of the skeletal modes to the transfer, represented by $z(T)$ and $z(T)\sqrt{2}$. If the atoms between which the tunneling takes place are known, or can be reduced to two possibilities, as is often the case, the transfer distance r_0 and thus the transfer parameter ρ can be estimated. Substitution

in Eq. (17-6) will typically give rise to a range of $z(T)$ values, with which the problem of the temperature dependence can then be tackled.

For this we require a specific form for the skeletal-modes amplitude $A(T)$. Elsewhere [14] we have shown that in simple cases we can deal with the skeletal vibrations $\Lambda(\mathbf{R})$ that modulate the equilibrium transfer distance r_0 in Eq. (17-3) by combining them into an *effective* mode, whose properties can be evaluated if a full force field calculation is available. Here we follow the common procedure of fitting the effective frequency and reduced mass of this mode to the observed kinetic parameters. Assuming that it is harmonic with an equilibrium position $R = R_0$, a frequency Ω and a reduced mass M , we derive values for these parameters from the observed temperature dependence of the transfer rate constants. In this approach the squared amplitude of the effective mode takes the form

$$A^2(T) = (\hbar/M\Omega) \coth(\hbar\Omega/2k_B T) \quad (17-7)$$

or, in dimensionless notation,

$$z(\xi) = \frac{\hbar/M\Omega}{2(a_{\text{eff}}^H)^2} \coth \xi \quad (17-8)$$

where $\xi = \hbar\Omega/2k_B T$ measures the vibrational energy in terms of the thermal energy and thus represents a scaled inverse temperature. The distribution $z(\xi)$ replaces $z(T)$ when the skeletal modes are represented by a single effective mode. Since this representation may tend to reduce skeletal-mode support, we expect that $z(\xi) \leq z(T)$.

Differentiating Eq. (17-5) with respect to the inverse temperature, and noting that $dT^{-1} = T^{-1} d \ln T^{-1}$, we obtain in dimensionless notation

$$-\frac{d \ln k^H(T)}{d \ln T^{-1}} \equiv \frac{E_a^H}{k_B T} = \rho \frac{z(\xi)F(\xi)}{[1 + z(\xi)]^2} + B(T) \quad (17-9)$$

$$-\frac{d \ln k^D(T)}{d \ln T^{-1}} \equiv \frac{E_a^D}{k_B T} = \rho \frac{2z(\xi)F(\xi)}{[1 + z(\xi)\sqrt{2}]^2} + B(T) \quad (17-10)$$

The scaled activation energies can be read directly off conventional Arrhenius plots since $d \ln T^{-1} = T d \ln T^{-1}$. The function $F(\xi) = \xi(\coth \xi - \tanh \xi)$, which varies smoothly from 0 to 1, as illustrated in Figure 17-1, depends only on the ratio $\hbar\Omega/k_B T$. It represents the dependence of the model on the assumption that the effect of skeletal vibrations on the transfer dynamics can be approximated by that of a single effective harmonic oscillator with frequency Ω collinear with the tunneling mode. It is very useful because, due to its limited range of permissible values, it provides a test for the compatibility of the data with the model. Apart from the upper limit $F(\xi) = 1$, there is a lower limit set by the physically acceptable range of values for the skeletal-mode frequency Ω .

Equations (17-9) and (17-10) express the dependence of the activation energies on the parameter ξ and thus on the temperature, information that cannot be obtained

Figure 17-1. Plot of the function $F(\xi) = \xi(\coth\xi - \tanh\xi)$

from the Arrhenius formula. Since both equations, considered as functions of $z(\xi)$, depend also on $F(\xi)$ and on $B(T)$, it is often more convenient not to use them directly but to transform to their ratio

$$\frac{E_a^D - \Delta E^{\text{eff}}}{E_a^H - \Delta E^{\text{eff}}} = 2 \left[\frac{1 + z(\xi)}{1 + z(\xi)\sqrt{2}} \right]^2 \quad (17-11)$$

and their difference

$$\frac{d \ln \eta}{d \ln T^{-1}} \equiv \frac{\Delta E_a}{k_B T} = z(\xi) F(\xi) \rho \left\{ \frac{2}{[1 + z(\xi)\sqrt{2}]^2} - \frac{1}{[1 + z(\xi)]^2} \right\} \quad (17-12)$$

where $\Delta E_a = E_a^D - E_a^H$. Using Eq. (17-6) we can eliminate ρ and obtain an equation that depends only on $z(\xi)$ and $F(\xi)$:

$$\frac{\Delta E_a / k_B T}{\ln \eta} = \frac{z(\xi) F(\xi)}{\sqrt{2} - 1} \left[2 \frac{1 + z(\xi)}{1 + z(\xi)\sqrt{2}} - \frac{1 + z(\xi)\sqrt{2}}{1 + z(\xi)} \right] \quad (17-13)$$

Thus Eqs. (17-11) and (17-13) have each only one extra parameter, ΔE^{eff} or $F(\xi)$. Moreover, these equations provide two new compatibility tests. From Eq. (17-11) it follows that $E_a^H \leq E_a^D \leq 2E_a^H$, and from Eq. (17-13) that $\Delta E_a / k_B T \leq 2 \ln \eta$. A disadvantage of Eqs. (17-11) and (17-13) relative to Eqs. (17-9) and (17-10) is that they may be more sensitive to the accuracy of the measured activation energies. Hence in practice it is prudent to use all four equations in the analysis.

Figure 17-2. Plot of $\Delta E_a/(\rho k_B T)$ (solid lines) and $(\ln \eta)/\rho$ (broken line) against $z(\xi)$ for $F(\xi) = 1$ (top) and 0.5 (bottom)

In Figure 17-2, we have plotted $\Delta E_a/(\rho k_B T)$ from Eq. (17-12) and $(\ln \eta)/\rho$ from Eq. (17-6) against $z(\xi)$ for $F(\xi) = 1$ and 0.5. In principle, the graph allows us to deduce both ρ , and thus the transfer distance r_0 , and $z(\xi)$, and thus the contribution of the effective mode to the tunneling, from the KIE and its temperature dependence, provided $F(\xi)$, which depends solely on the effective-mode frequency, can be estimated. If instead ρ can be estimated, we can deduce both r_0 and $F(\xi)$. Of course such estimates require reliable input values.

With Eqs. (17-6) and (17.9–17.13) we have the tools needed to test whether the temperature- and isotope-dependent rate constants k_{cat} of a given enzymatic reaction is compatible with the assumption that the rate is determined by a single tunneling step. If so, we can use the data to extract information on the vibrations governing the rates; if not, there is evidence that the rate is limited by more than one step. If the interfering slow steps are isotope-independent, they may only interfere with hydrogen and not with deuterium transfer, which is generally much slower. In that case the analysis should be mainly based on the deuterium data; an example of such a situation is treated in [8].

The treatment is readily extended to ratios of rate constants involving the same isotope but slightly different substrates or enzymes such as those obtained by mutations. In [8] this approach was applied to six mutants of the enzyme SLO1; the results of this analysis are summarized in Section 17.4.

17.3. PHYSICAL PARAMETERS

Two key parameters of the model are the equilibrium proton transfer distance r_0 , i.e. the distance between the proton equilibrium positions in the initial and final state, and the zero-point amplitude of the proton in the two states, $a_{\text{eff}}^{\text{H}}$. In practice, the transfer distance can be reasonably estimated from standard bond lengths and van der Waals radii, and the zero-point amplitude from standard XH-stretching potentials. Together they determine the distance parameter ρ defined by Eq. (17-4). For instance, the van der Waals radius of a donating methyl or methylene group is about 2 Å and that of an accepting oxygen atom about 1.4 Å. The standard CH-bond length is about 1.1 Å and the standard OH-bond length about 1.0 Å.

In the case of nonadiabatic transfer of the proton between a carbon and an oxygen center, we expect that hydrogen bonding will reduce the C...O distance and increase the OH-bond length. In the initial state we expect little hydrogen bonding, but in the final state, which contributes on an equal footing in the Golden Rule approach, the ionic structure may lead to strong bonding. Therefore we estimate the transfer distance (in Å) to be in the range $1.0 \leq r_0 \leq 1.2$. The zero-point amplitude a_0 for a harmonic oscillator with $m = 1$ and $\omega = 3,000 \text{ cm}^{-1}$ equals 0.105 Å. Taking into account that the oscillator will be anharmonic and that the anharmonicity will increase with increasing hydrogen bonding, we estimate $0.11 < a_{\text{eff}} < 0.14 \text{ Å}$. Combining these estimates, we arrive at $25 \leq \rho \leq 50$.

While hydrogen bonding reduces the tunneling distance, which favors transfer, it also tightens the C...O binding and thus increases the frequency Ω of the effective mode, which hinders its thermal excitation and thus has the opposite effect. Since the enzymes under investigation are the product of a long evolutionary process, the physical parameters governing the reaction are expected to be optimized such that at biologically relevant temperatures the contributions of the tunneling mode and the promoting mode are comparable, which leads to the estimate $z(\xi) \sim 1$. For the mass of the promoting mode we expect a relatively low value that may approach the lower limit $M \sim 1$; since the promoting mode is an effective mode this is not an absolute limit. The frequency of the C...H...O vibration is expected to be low in the initial state in which there will be little hydrogen bonding, but may be quite high in the final state, which is ionic. We estimate that the average of these values will remain below 400 cm^{-1} , which implies $F(\xi) \geq 0.5$.

For free radical reactions corresponding to neutral hydrogen transfer between methyl and/or methylene groups, we combine the van der Waals radius of about 2.0 Å for these groups with the CH-bond length of about 1.08 Å to obtain an equilibrium transfer distance $1.8 \leq r_0 \leq 1.9 \text{ Å}$. A similar value, namely 1.88 Å is obtained from data for the abstraction of a hydrogen atom by a methyl radical from the methyl group of methanol in methanol glass; using esr techniques, Doba et al. [15] measured a distance of 2.96 Å between the radical carbon and the nearest methyl proton. Since there will be no significant hydrogen bonding, the anharmonicity will be that of a standard CH bond; on that basis we estimate $a_{\text{eff}} \simeq 0.11 \text{ Å}$. These

estimates indicate that for neutral hydrogen transfer between two carbon centers ρ should be about 150. The absence of significant hydrogen bonding between the carbon centers also implies that the effective-mode frequency will be low in terms of the thermal energy. As a result, the effective-mode function $F(\xi)$ may be replaced by its maximum value $F(\xi) = 1$ for these transfers.

17.4. APPLICATION TO LIPOXYGENASE-1

The study of CH cleavage by soybean lipoxygenase-1 and six mutants by Klinman et al. [5, 6] represents the most complete experimental study presently available in the field of enzyme kinetics. Since a detailed analysis of these kinetic data was reported elsewhere [8], we restrict ourselves here to a brief summary to place it in the context of the other systems for which kinetic data are available. The observed rate constants and their temperature dependence are collected in Table 17-1. Of the mutations, the first four occur at the same site located at some distance from the reaction center [5, 6], while the last two occur at different and closer sites [5]. In all cases mutants differ only by an alkyl chain from the wild-type (WT) enzyme and from each other. The WT enzyme and the first two mutants have the same rate constants at $T = 298$ K and thus the same values for the parameters ρ and $z(298)$. It may appear therefore that these mutations have no effect on the transfer; however, the observed activation energies, which, admittedly have relatively large uncertainties, contradict this. The following two mutants have somewhat smaller rate constants, although the mutations are very similar as well as on the same site. Contrary to the suggestion of Meyer et al. [6], our analysis indicates that there are no prominent changes in the transfer distance. The final two mutants show a strongly reduced activity, but their KIEs are similar to those of the others. Here the lower activity implies an increase in the transfer distance, and thus in ρ , but the similar KIE indicates that the increased distance is accompanied by an increase in the contribution of the skeletal modes, $z(298)$. Specifically it indicates that r_0 must be

Table 17-1 Rate constants (in s^{-1}) and activation energies at $T = 298$ K of soybean lipoxygenase-1 and six mutants calculated by linear regression from the kinetic data reported as Supporting Information in [5, 6]

Enzyme	$\ln k^{\text{H}}$	$\ln k^{\text{D}}$	$\ln \eta$	$E_a^{\text{H}}/k_{\text{B}}T$	$E_a^{\text{D}}/k_{\text{B}}T$	$\Delta E_a/k_{\text{B}}T$
Linoleic/SLO1	5.7 ± 0.2	1.3 ± 0.3	4.4 ± 0.5	2.8 ± 0.5	5.3 ± 1.2	2.4 ± 1.7
Mutant I553A	5.7 ± 0.1	1.2 ± 0.4	4.5 ± 0.5	3.3 ± 0.7	8.6 ± 1.0	5.3 ± 1.7
Mutant I553L	5.8 ± 0.1	1.3 ± 0.3	4.5 ± 0.4	0.7 ± 1.2	6.7 ± 0.5	6.0 ± 1.7
Mutant I553V	4.3 ± 0.2	0.0 ± 0.4	4.3 ± 0.6	4.1 ± 0.9	8.3 ± 1.1	4.2 ± 2.0
Mutant I553G	4.1 ± 0.1	-1.2 ± 0.3	5.3 ± 0.4	0.0 ± 0.7	7.3 ± 0.7	7.3 ± 1.4
Mutant L546A	0.4 ± 0.2	-4.4 ± 0.4	4.8 ± 0.6	5.6 ± 0.4	9.3 ± 0.8	3.7 ± 1.2
Mutant L754A	-1.7 ± 0.3	-6.4 ± 0.4	4.6 ± 0.6	6.2 ± 0.8	8.9 ± 1.7	2.7 ± 2.5

Table 17-2 Model parameters calculated in [8] from the data listed in Table 17-1. Values of ρ in parentheses are chosen estimates

Enzyme	ρ	$z(298)$	$F(\xi)$	ξ	Ω/cm^{-1}	M/m^{H}
Lin./SLO1	(25)	0.44 ± 0.06	0.63 ± 0.10	0.87 ± 0.15	350 ± 80	14 ± 4
Mut. I553A	(25)	0.43 ± 0.06	~ 1	Small	Small	Large
Mut. I553L	(25)	0.42 ± 0.06	0.81 ± 0.07	0.56 ± 0.05	220 ± 30	32 ± 3
Mut. I553V	31 ± 2	0.60 ± 0.08	0.76 ± 0.10	0.65 ± 0.10	260 ± 50	17 ± 4
Mut. I553G	26 ± 2	0.38 ± 0.06	0.87 ± 0.10	0.45 ± 0.06	180 ± 40	52 ± 10
Mut. L546A	37 ± 3	0.65 ± 0.10	0.88 ± 0.10	0.44 ± 0.05	180 ± 50	31 ± 8
Mut. L754A	53 ± 4	1.0 ± 0.2	0.59 ± 0.15	0.93 ± 0.20	370 ± 80	6 ± 2

roughly proportional to the amplitude $A(T)$ of the effective mode, which supports the notion that this relationship is governed by hydrogen bonding in the final state of the transfer reaction.

Turning to the activation energy, we observe large experimental uncertainties and a loss of regularity. Application of Eqs. (17.9–17.13) leads to the conclusion that the data are not fully compatible with the model, a conclusion supported by the observation of very small and even vanishing activation energies for H, as opposed to D, transfer, which is not acceptable for room-temperature tunneling. It seems likely that these low values are due to contamination of the corresponding rates by isotope-independent slow steps in the reaction sequence. Such contaminations have indeed been observed in other reactions catalyzed by lipoyxygenase [16]. It is associated with fast tunneling steps, which appear to have evolved so as to reach a rate approaching that of other steps in the reaction. To complete the analysis we have therefore ignored the offending E_a^{H} values of the active enzymes. In Table 17-2 we summarize the quantitative results taken from [8].

17.5. APPLICATION TO FREE RADICAL TRANSFER

Most of the kinetic data on transfer of hydrogen transfer by a free-radical mechanism involve cobalamine (Cbl), a cobalt-centered complex which can release a ligand in the form of a free radical. A typical example is coenzyme B₁₂ in which the ligand is 5'-deoxyadenosyl (Ado). The radical thus formed abstracts a hydrogen atom from a carbon-hydrogen bond of the substrate, a process characterized by a large and strongly temperature-dependent KIE. The hydrogen abstraction step in methylmalonyl-coenzyme A mutase (MMCoAM) has been studied by Chowdhury and Banerjee [9]; the kinetic data relevant for our purpose are listed in Table 17-3 (Reaction 1). Since the transfer presumably occurs between a methylene group and a methylene radical, we expect the distance parameter ρ to much larger than in the case of SLO1, as discussed in Section 17.3. In view of the implied large transfer

Table 17-3 Kinetic parameters (T in K, k in s^{-1}) used in the analysis of hydrogen atom transfer

Nr.	Reactant	T	ΔT	$\ln k^{\text{H}}$	$E_a^{\text{H}}/k_B T$	$\ln \eta(T)$	$\Delta E_a/k_B T$	Reference
1	MMCoAM	293	15	5.3	31.8	3.8	6.9	9
2	(MeO)AdoCbl	353	40	—	—	2.5	3.5	10
3	NeopentylCbl	292	30	—	—	3.6	5.3	10
4	PheH	306	60	—	—	2.4	3.9	13
5	TyrH	306	60	—	—	2.6	4.7	13

distance, we expect the effective-mode frequency to be low and hence we set $F(\xi) \sim 1$. Furthermore, we assume that the reaction is not endothermic and involves little reorganization, in keeping with the accepted mechanism which suggests that the tunneling step is nonpolar and thermoneutral. Based on these assumptions, we can obtain values for the model parameters ρ and $z(293)$ from Eq. (17-6) and either Eqs. (17-9) and (17-10) or Eqs. (17-11) and (17-13). Although these two pairs of equations are equivalent, the first choice is the best in this case, since it is less dependent on the accuracy of the measured input parameters. The results, $z(\xi) = 2.66$ and $\rho = 160$, and $z(\xi) = 2.75$ and $\rho = 168$, are in excellent mutual agreement, thus supporting the validity of our assumptions. Using the default value $a_0 = 0.105 \text{ \AA}$, we derive an equilibrium transfer distance $r_0 = 1.9 \text{ \AA}$, a value in close agreement with the distance of $1.88 \text{ \AA}$ measured by Doba et al. for hydrogen transfer between a methyl radical and methanol; it also agrees with the value estimated from the van der Waals radius of $2.0 \text{ \AA}$ for methyl together with a CH-bond length of $1.08 \text{ \AA}$. The results again confirm the assumptions $\Delta E_0 + E_R \sim 0$ and $F(\xi) \sim 1$, implying a frequency $\Omega \leq 100 \text{ cm}^{-1}$ for the effective mode. It is worth noting that if we had used Eqs. (17-11) and (17-12) instead of (17-9) and (17-10), we would have obtained two conflicting values for $z(\xi)$ and thus r_0 , which can be traced back to the relative inaccuracy of ΔE_a , a value obtained as a small difference between two large numbers. Overall, we conclude that the model provides a good fit to the data, which confirms the rate-limiting character of the tunneling step and its adiabatic transfer mechanism.

A similar reaction, but without the enzyme, has been studied by Doll and Finke [10], whose substrate was the solvent, viz. ethylene glycol; the relevant kinetic data, listed in Table 17-3 as Reactions 2 and 3, are derived from product distributions. Strictly speaking, our equations, which neglect thermal excitation of CH and CD stretching modes, are not applicable to Reaction 2, which involves AdoCbl (as well as MeO-AdoCbl which yields similar results), because of the high temperature. We therefore consider only Reaction 3, in which Ado is replaced by neopentyl, which separates faster from Cbl. If we use Eq. (17-13), set $F(\xi) \sim 1$, and assume a thermoneutral reaction with minimal reorganization, we obtain $z(\xi) \simeq 2.4$ and, after substitution in Eq. (17-6), $\rho \simeq 130$, implying an equilibrium transfer distance

$r_0 = 1.7 \text{ \AA}$. This estimate indicates that the tunneling step is not enhanced by the presence of the enzyme, a conclusion similar to that of Doll and Finke [10].

Reactions 4 and 5 in Table 17-3 concern hydrogen transfer in phenylalanine hydroxylase; the listed kinetic data were measured by Pavon and Fitzpatrick [13] from product distributions. Application of Eq. (17-6) with $\rho \simeq 150$ yields $z(306) \simeq 3.4$ for Reaction 4 and 3.3 for Reaction 5, values that are similar to but somewhat larger than those for Reactions 1 and 3. The implied large transfer distances justify the assumption that $F(\xi) \sim 1$. Together with the relatively large values of $\Delta E_a/k_B T$, they indicate that the reaction proceeds by means of a free radical mechanism. Substitution of the observed values of $\Delta E_a/k_B T$ in Eq. (17-13) leads to $z(\xi) = 3.7$ and 13 for Reaction 4 and 5, respectively. Upon substitution in Eq. (17-6), the first value yields $\rho \simeq 170$, corresponding to a transfer distance $r_0 \simeq 1.9 \text{ \AA}$, similar to that found for coenzyme B₁₂. However, the second value is obviously much too large. In the absence of rate constants and activation energies, we have no means to trace back the cause of this discrepancy. Restricting ourselves therefore to Reaction 4, we propose that the model provides an acceptable fit to the data and suggests that the rate-limiting step corresponds to tunneling of a neutral hydrogen atom between two carbon centers.

17.6. APPLICATION TO METHYLAMINE DEHYDROGENASE

Scrutton and coworkers [11, 12] have studied the oxidative demethylation of primary amines to form aldehydes and ammonia by methylamine dehydrogenase (MADH) and also by aromatic amine dehydrogenase (AADH), which shows much the same behavior. In these reactions a proton is abstracted by an active-site base from an iminoquinone intermediate while an electron is transferred to a tryptophyl-quinone cofactor. Most of these reactions exhibit KIEs between 5 and 20 at 298 K with a very weak temperature dependence, although the rate constants show activation energies of more than 10 kcal/mol. The authors assumed that the proton-transfer step is rate-determining. However, they report that the kinetics do not follow the Michaelis-Menten equation; specifically the association and dissociation rate constants of the enzyme-substrate complex, denoted by k_1 and k_{-1} in Eq. (17-1), are subject to KIEs of the same general magnitude as k_{cat} . Our earlier efforts [1, 7] to fit the data to a tunneling model were unsuccessful.

Our present analysis starts with the basic substrate methylamine [11], for which a KIE of 16 ($\ln \eta = 2.8$) was observed. Application of Eq. (17-6) to the estimated range of transfer distances, represented by $25 \leq \rho \leq 50$, yields $0.76 \leq z(298) \leq 1.43$. These numbers are comparable to those for SLO1 and indicate that $F(\xi)$ will be smaller than unity. Substitution in Eq. (17-11) together with the observed activation energies $E_a^{\text{H}}/k_B T = 17.6$ and $E_a^{\text{D}}/k_B T = 18.2$ yields effective endothermicities in the range $16.6 \geq E^{\text{eff}}/k_B T \geq 15.9$. These numbers are suspect, however, because they give rise to very small activation energies, not only for H transfer (less than $2k_B T$, as in SLO1 reactions, but also for D transfer (less than

$2.5k_B T$. If we substitute the deduced range of $z(T)$ values, interpreted as $z(\xi)$, in Eq. (17-13), we obtain a range of values of $F(\xi)$ of $0.21 - 0.17$, implying an effective-mode frequency Ω well above the proposed limit of 400 cm^{-1} , corresponding to $F(\xi) \sim 0.5$. Use of Eqs. (17-9) and (17-10) instead of (17-11) and (17-12) confirms that there is no combination of acceptable values for E^{eff} and the temperature dependence of the KIE that can reproduce the experimental data. It follows that these data are not representative of the properties of a single tunneling step. This is likely to be related to the observation that the kinetics does not follow the Michaelis-Menten equation (17-1).

Much the same holds for the data reported for a number of AADH reactions [12]. However, the MADH reaction in which methylamine is replaced by ethanolamine [12] is an exception. The latter reaction exhibits the same KIE as the methylamine reaction, implying the same range of $z(298)$ values. While it has nearly the same activation energy for H transfer (17.6 against 17.9 in units of $k_B T$), its activation energy for D transfer is considerably larger (20.2 against 18.5 in units of $k_B T$). As a result the value of E^{eff} is much reduced, namely to the range $11.7 \geq E^{\text{eff}}/k_B T \geq 8.6$, implying acceptable values for the activation energies. If their difference is substituted in Eq. (17-13), it yields acceptable values for $F(\xi)$, namely a range of $0.96-0.74$, corresponding to a range of $100-280\text{ cm}^{-1}$ for the effective-mode frequency Ω . Hence it appears that a single tunneling step determines the rate of the reaction of ethanolamine, in contrast to that of methylamine. This may be related to the fact that the ethanolamine reaction is considerably slower and thus less likely to suffer interference from isotope-independent steps.

17.7. DISCUSSION

It has been our purpose to show that an investigator of enzymatic CH-cleavage reactions, who is likely to encounter large kinetic isotope effects, need not be limited to the conclusion that the reaction proceeds by quantum-mechanical tunneling. With the use of the proper tools, a good deal of additional information can be gathered about the mechanism of the reactions and the vibrations governing their rates. In this contribution we offer such a tool in the form of an analytical model that is simple to use and can be applied directly to the data in the form in which they are traditionally reported. Our model is meant to replace the Arrhenius equation, which is manifestly invalid for tunneling reactions, and the Bell model, which overlooks the effect of skeletal vibrations on tunneling. To demonstrate the usefulness of the model, we have applied it to a representative set of enzymatic reactions for which KIEs and their temperature dependence are available.

From our analysis of these reactions, we conclude that not all of them are compatible with the model. While the model in the form used here is very basic, it is structured in such a way that incompatibility is readily recognized and can be traced back to (a specific subset of) the data. As the applications show, the data recognized as problematic tend to suffer from other problems as well. In general,

incompatibility serves as a warning that the kinetics of these reactions may not be governed by a single rate-limiting tunneling step, but could, e.g., suffer from interference from slow isotope-independent steps. If the data pass the compatibility test, they can offer information on such quantities as the hydrogen transfer distance, the endothermicity, if any, the contribution of skeletal modes to the transfer, the properties of these modes as well as of the tunneling mode, the absence or presence of hydrogen bonding, and the effect of mutations, if any. To derive these properties from the observed kinetic parameters, some empirical input is required such as the van der Waals radii of the atomic centers between which the hydrogen is transferred and relevant bond lengths, but even with a minimum of input, information can be extracted that cannot be obtained from the Arrhenius equation and the Bell model. None of this requires any computational effort, although the availability of theoretical input or the use of more elaborate versions of the model, where the data warrant it, is likely to improve the accuracy of the results.

REFERENCES

1. Siebrand W, Smedarchina Z (2006) In: Kohen A, Limbach H-H (eds) *Isotope effects in chemistry and biology*. CRC Press, Baton Rouge
2. Bell RP (1980) *The tunnel effect in chemistry*. Chapman & Hall, London
3. Siebrand W, Wildman TA, Zgierski MZ (1984) *J Am Chem Soc* 106:4083–4089; *ibid* 4089–4095
4. Kuznetsov AM, Ulstrup J (1999) *Can J Chem* 77:1085–1096
5. Knapp MJ, Rickert KW, Klinman JP (2002) *J Am Chem Soc* 124:3865–3874
6. Meyer MP, Tomchick DR, Klinman JP (2008) *Proc Natl Acad Sci USA* 105:1146–1151
7. Siebrand W, Smedarchina Z (2004) *J Phys Chem* 108:4185–4195
8. Siebrand W, Smedarchina Z (2010) *J Phys Org Chem* 23: issue 7
9. Chowdhury S, Banerjee R (2000) *J Am Chem Soc* 122:5417–5418
10. Doll KM, Finke RG (2003) *Data Inorg Chem* 42:4849–4857
11. Basran JB, Sutcliffe MJ, Scrutton NS (1999) *Biochemistry* 38:3218–3222
12. Basran JB, Patel S, Sutcliffe MJ, Scrutton NS (2001) *J Biol Chem* 276:6234–6242
13. Pavon JA, Fitzpatrick PF (2005) *J Am Chem Soc* 127:16414–16415
14. Smedarchina Z, Siebrand W (1993) *Phys Chem* 170:347–358
15. Doba T, Ingold KU, Reddoch AH, Siebrand W, Wildman TA (1984) *J Chem Phys* 8:6622–6630
16. Jacquot C, Wecksler AT, McGinley CM, Segraves EN, Holman TR, van der Donk WA (2008) *Biochemistry* 47:7295–7304