J. Phys. Chem. B **2007,** *111,* 6643−6654

H and Other Transfers in Enzymes and in Solution: Theory and Computations, a Unified View. 2. Applications to Experiment and Computations[†]

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Received: February 26, 2007; In Final Form: March 30, 2007

Equations obtained in part I for the free-energy barrier to one-step enzymatic reactions between bound reactants are discussed. The rate is expressed in terms of λ_o (protein reorganization energy), $\Delta G°$ (standard free energy of reaction of the H-transfer step), bond breaking/bond forming term, w (work terms), and H-transmission property. Two alternative approximations for the coupling of the bond breaking/bond forming and protein are distinguished experimentally in favorable cases by the $\Delta G°$ where the maximum deuterium kinetic isotope effect occurs. Plots of log rate versus $\Delta G°$ and properties such as ΔS^* and $\Delta S°$ are discussed. The weak or zero T-dependence of the kinetic isotope effect for wild-type enzymes operating under physiological conditions is interpreted in terms of vanishing (or isotopically insensitive) w plus transfer from the lowest H-state. Static and dynamic protein flexibility is discussed. While the many correlations accessible for electron transfers are not available for H-transfers in enzymes, a combination of experiment, computation, and analytical approaches can assist in evaluating the utility of the present equations and in suggesting further experiments and computations. A protein reorganization energy λ_o is obtained in the literature from the extended valence bond formalism where diabatic electronic states are used. A method is suggested for extracting it when instead a bond distance difference coordinate is used. The results may provide a bridge between the two approaches.

1. Introduction

The field of enzyme catalysis has been particularly active in recent years with many developments, both experimental and computational described, for example, in reviews.[1−8] In part I we considered the rate of transfer of an H^+, H^-, $H^•$, or other groups in enzymes.[9,10] An equation was given for the reaction rate from bound reactants (DH + A) to bound products (D + HA). The theoretical expression was a combination of an earlier equation[11] for the bond breaking−bond forming reaction and a quadratic expression[12,13] for the "reorganization" of the protein to reach the transition state. The DHA reacting pair is commonly defined to be the atoms directly or closely involved in the covalent bond breaking−bond forming reaction, frequently some 50 or so atoms. The remaining part of system, namely, the rest of the substrate−cofactor complex, the enzyme as a whole, and the surrounding solution, was abbreviated as "protein" and is denoted throughout by "prot". In QM/MM computations in the literature quantum mechanics is used for DHA and molecular mechanics for the rest of the system.

Two independent variables m and n were used in Part I to characterize the reaction (model A). We consider now a model, model B, in which the progress along the bond breaking−bond forming coordinate and that along the protein reorganization coordinate are tightly coupled so that only a single variable n is used. Experimental data and computations for enzymes are examined in terms of the equations, and further experiments and computations are suggested.

Experimental data for enzymatic catalysis are much more limited than are the data for electron transfers. For example, there are no data on "self-exchange" reactions for enzymatic H transfers, apparently no data on charge-transfer spectra due to transfer of an H, and there are usually data only for a relatively narrow range of $\Delta G°$ for the H-transfer step. It is not unexpected, therefore, that now a combined experimental/computational analysis can be even more useful for interpreting experimental data and for suggesting additional studies. In electron transfers, in contrast, much of the theory could be and was tested experimentally by its predicted[14] correlations between independent sets of data.[15]

In the present paper we focus on H transfers $H^•$, H^+, H^- in enzymes. The paper is subdivided as follows: Equations for the free-energy barrier for the H transfer step in the reaction are given in section 2. Equations for the rate constant are given in section 3 and for the kinetic isotope effect (KIE) for deuterium and ^{13}C, including the dependence on $\Delta G°$, in section 4. The difference in the dependence of the position of the maximum deuterium KIE versus $\Delta G°$ plot in the two models is noted. Examples of experimental data and computations are discussed in section 5 for free energies of activation, reaction rates, deuterium, and ^{13}C KIE, pressure effects on these KIEs, and the weak or zero temperature dependence of the deuterium KIE in certain enzymes. While the extraction of the reorganization parameter λ from empirical valence bond (EVB) based calculations is well-known, the approximations of the method lending itself quite naturally to that formalism, λ has not been extracted from the formalism based upon a bond-length difference reaction coordinate. It is not even known whether the concept is viable with such a formalism. A method is suggested in section 6 for extracting a λ_o from it and for examining some of the assumptions. Concluding remarks are given in section 7.

2. Theoretical Equations for the Free-Energy Barrier

Model A. Two Reaction Progress Coordinates. The free-energy barrier to the reaction ΔG^* for this model, for a

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[†] Part of the special issue "Norman Sutin Festschrift".

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10.1021/jp071589s CCC: $37.00

separation distance R of the reacting pair in the bound complex, was written as[9]

$$\Delta G^* = w^r(R) + \Delta G^*(R) \tag{1}$$

where

$$\Delta G^*(R) = \Delta E^*_{DHA}(R) + \Delta G^* \text{ (prot)} \tag{2}$$

and the contribution from the DHA reacting pair is

$$\Delta E^*_{DHA}(R) = \frac{\lambda_i}{4} + \frac{\Delta E^\circ_{DHA}(R)}{2} + \frac{\lambda_i}{4 \ln 2} \ln \cosh y \tag{3}$$

This $\Delta E^*_{DHA}(R)$ includes the interactions within DHA. The λ_i is the bond "reorganization energy," and because of a symmetrization approximation[11] used in obtaining eq 3, it is a symmetrized property, a mean of the properties of the two chemical bonds (eq 8c of ref 11); y is given by

$$y = (2\Delta E^\circ_{DHA}(R) \ln 2)/\lambda_i \tag{4}$$

where $\Delta E^\circ_{DHA}(R)$ is the DHA contribution to the standard free energy of reaction ΔG° (R) of the H-transfer step at R:

$$\Delta G^\circ(R) = \Delta E^\circ_{DHA}(R) + \Delta G^\circ \text{ (prot)} \tag{5}$$

The $\Delta E^*_{DHA}(R)$ in eq 3 has the expected asymptotic behavior, namely, that as $\Delta E^\circ_{DHA}(R) \rightarrow -\infty$, $\Delta E^*_{DHA}(R) \rightarrow 0$ and as $\Delta E^\circ_{DHA}(R) \rightarrow +\infty$, $\Delta E^*_{DHA}(R) \rightarrow \Delta E^\circ_{DHA}(R)$. In comparison with electron transfer, H-transfer is very sensitive to the D−A distance, and appears in the λ_i, in eqs 3 and 4, and later in the nuclear tunneling contribution.

ΔG^* (prot) in eq 2 is a contribution to the free-energy barrier $\Delta G^*(R)$ and arises from the changes in interaction of the charges and dipoles in the protein with each other and with the DHA. It was written as[9]

$$\Delta G^* \text{ (prot)} = [\lambda_o + \Delta G^\circ \text{ (prot)}]^2/4\lambda_o \tag{6}$$

where λ_o is the protein "reorganization energy". ΔG° (prot) is the contribution from the interactions within the protein and with DHA to the free energy of reaction $\Delta G^\circ(R)$. This equation, frequently with λ_o and ΔG° (prot) replaced by a total λ an ΔG°, is often used in phenomenological and other analyses of enzymatic free-energy barriers. The quadratic nature of eq 6 has a statistical mechanical origin in a linear response approximation.[16,17] A linear response formalism has been used to simplify QM−MM computations for a nonenzymatic reaction and appears via a cumulant expansion truncated at the second-order term.

An equation similar to eq 6 was derived initially for electron transfers[11−15] but with λ_o and ΔG° (prot) replaced by a total λ and ΔG° , where λ denotes ($\lambda_o + \lambda_i$). It is frequently used as such for H transfers, with λ and ΔG° being the quantities for the entire protein plus solute system. It is applied particularly when the empirical valence bond method (EVB)[6,16,18] is used. Relationships of several approaches are discussed in ref 19.

The $\Delta G^\circ(R)$ in eq 5 is related to the overall standard free energy of reaction ΔG° of the H transfer step in the bound complex:

$$\Delta G^\circ(R) = \Delta G^\circ + w^p(R) - w^r(R) \tag{7}$$

where $w^r(R)$ is the work done (electrostatic work, for example, or steric restrictions) to bring the two reactants, D and HA, from their equilibrium positions in the bound complex to a typical separation distance R at which the actual change in DH bond length begins in the H transfer, and $w^p(R)$ is the corresponding quantity for the products, taking for simplicity the actual change in HA bond length to begin at the same R. Technically, $w^r(R)$ is the part of the free-energy barrier in eq 1 that is not overcome by a favorable $\Delta G^\circ(R)$, and $w^p(R)$ in the corresponding quantity for the reverse reaction. Included in w^r (w^p) are any reorientation of the two reactants (products). Any change of zero-point energy of the H vibrations (stretching and two bending vibrations) along the DHA coordinate is taken, for notational simplicity, as being included in the DHA QM term. The w's represent the "gating" term discussed in the literature. They are discussed later in this paper.

In addition to the H/D tunneling considered later any R-dependent term appearing in eqs 1−7, such as w^r, may be isotopically sensitive, since R may be isotope-dependent. When one bond is broken and another is formed, ΔG° and ΔE°_{DHA} are expected to have little isotopic sensitivity, λ_o is expected to be isotopically insensitive, and λ_i may be isotopically sensitive since it now contains the effect of zero-point energy change along the reaction coordinate. The tunneling factor given later is of course isotopically sensitive.

For later use in expressions for the kinetic isotope effects and in analysis of computations we recall that a dimensionless bond distances coordinate n in the TS satisfied a symmetrized equation[9] when no other contributions to the free-energy barrier of the reaction were present.

$$(\lambda_i/4 \ln 2)\ln[n/(1-n)] = \Delta E^\circ_{DHA}(R) \tag{8}$$

Here, n is the bond order of the newly formed bond and equals $^1/_2$ when $\Delta E^\circ_{DHA}(R) = 0$, and is restricted to lie in an interval $0 < n < 1$. For small $\Delta E^\circ_{DHA}(R)/\lambda_i$, eq 8 yields[9]

$$n = 1/2 + (\Delta E^\circ_{DHA}(R) \ln 2)/\lambda_i + ... \tag{9}$$

Equations 3 and 6 were given in the present symmetrized form to provide simple looking expressions for treating the experimental data, as in earlier work on bond forming/bond breaking reactions[11] and on electron-transfer theory.[14] Although the key equations, eqs 3 and 4, have the expected asymptotic behavior when $\Delta E^\circ_{DHA}(R) \rightarrow \pm\infty$, that is, as $n \rightarrow 0$ or 1, eq 3 was derived for a reaction that proceeds from infinite separation of reactants to infinite separation of products. In terms of the actual dependence on n, eq 9 is best used in the vicinity of $n = {^1/_2}$, when compared with computations as a function of n. Models more general in functional form can be introduced, based on proceeding from a finite DH-A distance in the bound complex to a finite D-HA distance in that complex, as in section 6.

Model B. A Single Reaction Progress Coordinate. We recall how eqs 2−6 are derived: The TS for the DHA in model A was determined using a maximization along a reaction coordinate n for DHA. A different method was used for the protein coordinate m, one similar to that used in electron transfer,[14] namely, equating the free energies of the reactant and product states in the TS.[11−15] Specifically, if we write the free energy before the H-transfer as $m^2\lambda_o + E_{DHA}(n,R)$ and as $(1-m)^2 \lambda_o + \Delta G^\circ \text{ (prot)} + E_{DHA}(n, R)$ after the transfer, then upon equating the two, m was obtained from

$$m^2 \lambda_o - (1-m)^2 \lambda_o = \Delta G^\circ \text{ (prot)}, \quad \text{i.e.,} \quad -(1-2m)\lambda_o = \Delta G^\circ \text{ (prot)} \tag{10}$$

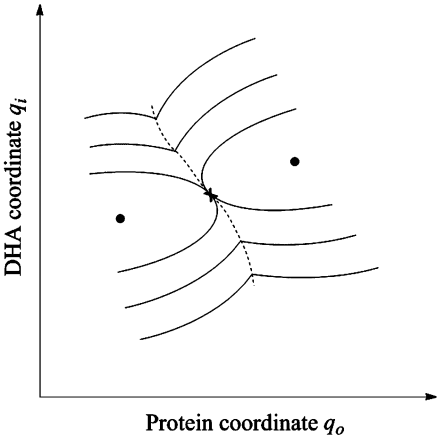

Figure 1. Schematic contours of free energy in a DHA coordinate (q_i) vs protein reorganization coordinate (q_o) space. The dotted line is the set of coordinates constituting the TS.

With this value of m the protein contributions in eqs 2 and 6 were obtained.

For model B, eq 1 applies as before, but instead of eqs 2−6 we now consider the case where the two coordinates are so tightly coupled that a single coordinate suffices to describe the reaction, just as a single Lagrangian multiplier m sufficed in the electron-transfer problem involving both solvent and solute coordinates.

Free-energy contours are depicted in Figure 1. In model B the n denotes a coordinate along the reaction path, a path that transverses the TS, the dotted line in Figure 1. When n changes both the DHA and protein coordinates, q_i and q_o there, change. To illustrate the issue, we consider first a harmonic model for both contributions. Equation 10 is again obtained, but with λ_o replaced by $\lambda_o + \lambda_i$ and with $\Delta G°$ (prot) replaced by $\Delta G°(R)$. Equation 6 then follows, with these replacements. Thus, the two contributions are tightly coupled now and the result is different in structure from that in model A, in that there is no longer a simple sum of two separate terms, such as the type of sum in eq 2.

An aim in model B will be to retain the tightly coupled form for the two contributions but still have a DHA term of the functional form that led to eq 3 in model A, instead of being a harmonic expression. To do so we make use of a device that places the two contributions to the free-energy barrier on the same footing, rather than obtaining one by a maximization and the other separately by equating two free energies.

In model B we first employ the general symbol, $E_{\mathrm{DHA}}(n,R)$, for the DHA term instead of the specific form used to derive eq 3, and then we use a single coordinate n so as to satisfy a single equation for n in the TS. One approach, prompted in part by the second half of eq 10, is to choose n in the TS to satisfy

$$\frac{\partial}{\partial n}E_{\mathrm{DHA}}(n,R) + \lambda_o(1-2n) + \Delta G°(R) = 0 \qquad (n \text{ in TS}) \quad (11)$$

This equation for n in the TS is equivalent to finding the maximum of an expression

$$\Delta G(n,R) = E_{\mathrm{DHA}}(n,R) + \lambda_o n(1-n) + n\Delta G°(R) \qquad (n \text{ in TS}) \quad (12)$$

It satisfies the initial condition $\Delta G(0,R) = 0$, taking $E_{DHA}(0,R) = 0$. This equation and eq 11 are an interpolation in that the maximum of $\Delta G(n,R)$ gives the correct values of n in the TS in the two limits, one where $E_{\mathrm{DHA}}(n,R) = 0$, and the other where $\lambda_o = 0$. As noted in the equation, it is intended only to determine n in the TS and not to serve as a profile of the free-energy change along the reaction coordinate n.

For concreteness two examples for $E_{\mathrm{DHA}}(n,R)$ are considered, one given later in section 6. We first write $E_{\mathrm{DHA}}(n,R)$ in the form in eq 12. $\partial\Delta G(n,R)/\partial n = 0$; then yield the value of n in the TS,

$$-\left(\frac{\lambda_i}{4\ln 2}\right)\ln[n/(1-n)] + \lambda_o(1-2n) + \Delta G°(R) = 0 \qquad (n \text{ in TS}) \quad (13)$$

This equation reduces to previous expressions[9,10] for the two individual results for DHA and prot, when λ_o or λ_i vanishes, respectively. For example, when $\lambda_o = 0$, eq 13 reduces to eq 8.

For the present case eq 12 yields for n in the TS

$$\Delta G(n,R) = -\left(\frac{\lambda_i}{4\ln 2}\right)[n\ln n + (1-n)\ln(1-n)] + \lambda_o n(1-n) + n\Delta G°(R) \qquad (n \text{ in TS}) \quad (14)$$

The free-energy barrier for this particular form for $E_{\mathrm{DHA}}(n,R)$ in model B obtained from eqs 13−14 gives

$$\Delta G^*(R) = -\frac{\lambda_i}{4\ln 2}\ln(1-n) + n^2\lambda_o \qquad (n \text{ in TS}) \quad (15)$$

where n is the value in the TS and is the solution of eq 13. $\Delta G^*(R)$ is introduced into eq 1 for the overall free-energy barrier ΔG^*.

While $\Delta E°_{\mathrm{DHA}}(R)$ no longer occurs explicitly in eqs 13 and 15, and so no longer solely controls the n for DHA in the TS for model B, it does contribute to the driving force $\Delta G°(R)$ in eq 2. A difference in the TS from that in model A is immediately apparent: when a system has a large $|\Delta E°_{\mathrm{DHA}}(R)|$ in model A its TS is highly asymmetric and n is very different from $^1/_2$. However, in model B if $|\Delta G°$ (prot)$|$ is large enough to compensate for $\Delta E°_{\mathrm{DHA}}(R)$, such that $\Delta G°(R) \approx 0$, the TS would instead be nearly symmetric, that is, $n \cong {}^1/_2$. A possible example of each model in the computational literature is noted in section 3, where a method is suggested for distinguishing models A and B experimentally under favorable circumstances, namely, determining experimentally whether the maximum of the deuterium KIE occur at $\Delta G°(R) = 0$ or at $\Delta E°_{\mathrm{DHA}} = 0$.

When $\Delta G°(R)$ is small, eqs 13 and 15 can be expanded about $n = {}^1/_2$ to yield for the TS

$$n = {}^1/_2 + \Delta G°(R)/2\Lambda_2 + ... \quad (16)$$

and then

$$\Delta G^* = w^r + \frac{\lambda_o + \lambda_i}{4} + \frac{\Delta G°(R)}{2} + \frac{\Lambda_1(\Delta G°(R))^2}{4\Lambda_2^{\,2}} + ... \quad (17)$$

where

$$\Lambda_1 = \frac{\lambda_i}{\ln 2} + \lambda_o,\ \Lambda_2 = \frac{\lambda_i}{2\ln 2} + \lambda_o \quad (18)$$

The R-dependent terms in eqs 16−18, principally w^r and w^p, may again be isotopically sensitive, depending on whether R is isotopically sensitive. (If the total zero-point energy does not change along the reaction coordinate before R is reached, then

R will not be isotopically sensitive.) The H-transfer transmission factor, discussed later, is again isotopically sensitive. When there is no change in the total number of bonds in the H transfer step $\Delta G°(R)$ is expected to be isotopically insensitive.

We have focused on a bond order n as a reaction coordinate in model B. One can also use the vertical energy difference in one EVB treatment or that of the two lowest adiabatic electronic states as a reaction coordinate. To do so the latter requires a calculation of the two lowest adiabatic electronic states of DHA and protein, rather than only the lowest. The vertical energy difference ΔE of the two diabatic states has been a useful reaction coordinate. Whether the vertical energy difference of two adiabatic states is also useful remains to be explored. Instead, for a treatment using adiabatic states it may be necessary to first transform them into diabatic states to define a suitable ΔE coordinate.

3. General Remarks on k_{rate} and on ΔS^*

For the moment we neglect nuclear tunneling and treat it later. The first-order rate constant of the reaction for a one-step process, in the present case of a bound pair in the complex, is written in TS theory as $k_{\text{rate}} = (kT/h)\exp(-\Delta G^{\ddagger}/kT)$, where $\Delta G^{\ddagger}$ is the free energy of activation. We introduce a related quantity, the free-energy barrier ΔG^*, equal to $\Delta G^{\ddagger} - kT\ln kT/h\nu$, where $kT/h\nu$ is the classical limit of the partition function, of the reactants' relative motion, treated approximately as a harmonic oscillator with a vibration frequency ν; ν is perhaps $\sim 10^{13}$ s^{-1}. We thus obtain

$$k_{\text{rate}} = \nu \exp(-\Delta G^*/kT) \tag{19}$$

The ΔG^* is written in eq 1 as the sum of w^{r} and $\Delta G^*(R)$. We recall that for notational simplicity any reorientation requirements for reaction are incorporated into w^{r}.

An equation for the corresponding entropic contribution to the reaction barrier ΔS^* and for the standard entropy of reaction at R, $\Delta S°(R)$, is obtained, in principle, from

$$\Delta S^* = -\,\partial\Delta G^*/\partial T, \quad \Delta S°(R) = -\partial\Delta G°(R)/\partial T \tag{20}$$

The rate constant written in an Arrhenius form is

$$k_{\text{rate}} = A\exp(-E_{\text{a}}/kT) \tag{21}$$

where the activation energy E_{a} is defined experimentally as $-k\partial\ln k_{\text{rate}}/\partial(1/T)$. From eqs 19−21 we then have $E_{\text{a}} = \Delta G^* + T\Delta S^*$ and so equals ΔH^*. Thus, when ΔG^* is temperature-dependent $E_{\text{a}} \neq \Delta G^*$. This fact, well-known in the literature and discussed in ref 15, has been occasionally overlooked. It can be shown that ΔS^* differs from the entropy of activation $\Delta S^{\ddagger}$ by a small amount, k. From eqs 19−21 we have

$$A = \nu\exp(\Delta S^*/k), \quad E_{\text{a}} = \Delta H^* \tag{22}$$

In passing, we note that the heat of activation $\Delta \mathrm{H}^{\ddagger}$, obtained from $\Delta G^{\ddagger}$ in the standard way, is related to E_{a} by $E_{\text{a}} = -k\partial\ln k_{\text{rate}}/\partial(1/T) = kT + \Delta\mathrm{H}^{\ddagger}$. Thus it differs from the ΔH^* in eq 22 by the small amount kT.

Other things being equal, the more negative the $\Delta S°$ the more negative the ΔS^*, as discussed in Marcus and Sutin[15] for electron transfers. Because of the dependence of ΔG^* on $\Delta G°$, similar remarks for ΔS^* apply to the H-transfers. Nuclear tunneling can affect the numerical values of both A and E_{a}. Any steric requirements present in the w^{r} enter via the $-\partial w^{\text{r}}/\partial T$ that contributes to ΔS^* (cf. eq 1).

When there are two or more reaction steps rather than one, and when the reaction step from the reactants to a reaction intermediate is rate determining, the overall $\Delta G°(R)$ is not the relevant quantity to correlate with the observed rate but rather the $\Delta G°(R)$ for the rate-determining step. For example, if variation of the overall $\Delta G°(R)$ has little effect on the first step, but the first step is the slow one, the rate plotted versus $\Delta G°$-(R)will reach a limiting low value, and the residual barrier will be attributed erroneously to a large w^{r}. A specific likely example is considered later. Again, in this two or more step reaction, it can also happen that an overall $\Delta G°$ changes little, but the ΔG^* for the rate-determining formation of the intermediate changes much more. In that case an abnormally large apparent slope of a $\Delta G^*(R)$ occurs over all $\Delta G°(R)$.[18] Another source of unusual slopes of a ΔG^* versus $\Delta G°$ plot occurs when a substrate or cofactor is varied and not only $\Delta G°$ but also λ_{o} or λ_{i} changes, an occurrence that complicates the interpretation and needs to be incorporated.[20−25]

A common theme in discussions of enzyme catalysis is the question of dynamical versus statistical effects. We recall that in a classic paper in 1938, Wigner pointed out that within the framework of classical mechanics (1) if there is a hypersurface in phase space such that there are no recrossings of it by classical trajectories and (2) if there is a thermal equilibrium statistical mechanical distribution in the reactants' phase space, then TS theory would be exact (exact within the framework of classical mechanics). The TS rate in this framework is a maximum and the correct rate is obtained by corrections for recrossings. It is common therefore to search for recrossings in the computations of rates.

For simplicity of terminology we shall term deviations from TS theory due to recrossings as being a dynamical effect, even though the underlying framework is, via Wigner, though not in the original 1935 derivation of Eyring or of Evans and Polanyi, dynamical. Their derivation was statistical. Typically, this dynamical correction factor in current calculations of enzymes has been close to unity. More precisely there are few recrossings within the time scale used for the trajectories (nonequilibrated trajectories, of course). However, these classical trajectories are typically for short times. If flexibility of the protein becomes important, recrossings for a slow reaction coordinate may occur on a much longer time scale, and if so, might not be observed on the relatively short time scale where recrossings are typically studied in computations.

4. Theoretical Equations for Deuterium and ^{13}C Kinetic Isotope Effects

General Remarks. Extensive calculations in the literature of the H/D/T KIE and nuclear tunneling in these reactions are described in recent reviews.[1−8,17,26] The deuterium KIE may play an added role, it will be seen later in this section, in distinguishing between the two models A and B. Typically the focus in the computational literature has been on the deuterium KIE rather than on ^{13}C. Although the ^{13}C KIE is accurately measurable experimentally, it is very small and so perhaps not easily calculated in numerical computations for large enzyme systems. An approximate functional form for the dependence of each KIE on asymmetry ($n \neq {}^{1}/_{2}$) is noted below. The present analysis is intended to complement and utilize the extensive and invaluable computational studies of the deuterium KIE.

Deuterium Kinetic Isotope Effect. Considering first the "symmetric" case ($\Delta E^{\circ}_{\text{DHA}} = 0$ in eq 9 or $\Delta G°(R) = 0$ in eqs 13−15) for the present discussion an approximate expression for the rate constant was given by[9,25]

$$k_{\mathrm{rate}}(R) \cong \nu \int P(E)\exp(-E/kT)\,\mathrm{d}(E/kT) \qquad (23)$$

where $P(E)$ is the probability of reaction at energy E, and ν is again the vibration frequency for the relative motion of DH and A. For the symmetric case, $n = {}^1/_2$, and the $P(E)$ for a $0 \rightarrow 0$ transition of the H state in a triatomic system is denoted by $P_{00}(E)$. It is given by eq 27 of Part I. In Figure 2 a nontunneling (α) path is depicted using the usual mass-weighted coordinate axes used in Part I and extensively used in the literature in simplified approximate analyses of three-center reactions. In the tunneling regime, represented by a β path in Figure 2, the equation for $P_{00}(E)$ reduces to

$$P_{00}(E) = A' \exp(-2K(R_{\mathrm{o}}) \exp(-\Delta G^*/kT) \qquad (24)$$

where $K(R)$ is the tunneling phase integral, $\int_{\theta_1}^{\theta_2}|p_\theta(R)|\,\mathrm{d}\theta/\hbar$, at a distance R of closest approach, from a point X′ ($\theta = \theta_1$) to Y′ ($\theta = \theta_2$) in Figure 3. (Polar coordinates R and θ are introduced in the Figures 2−4 but are not indicated explicitly.) The pre-exponential factor A' is given by eq 30 of Part I and is often neglected, though there is no need to do so. The P's in eqs 23−24 tacitly contain a term w^{r} via the ΔG^* in eq 3 that by definition contains orientation and distance R effects. This factor $\exp(-w^{\mathrm{r}}/kT)$ could be expressed in more formal statistical mechanical terms, but we have simplified the notation. Equation 24 takes no account of a possible "resonance" effect between an H-state in the donor-enzyme DH valley and an H-state in acceptor-enzyme HA valley in the potential-energy surface.

Equation 24 applies to the symmetric case ($n = {}^1/_2$) and so gives the value where the tunneling contribution is a maximum. A possible functional form that can be explored when the data are for an asymmetrical system was suggested in terms of the value of n in the TS:[9,10]

$$k_{\mathrm{H}}/k_{\mathrm{D}} \cong (k_{\mathrm{H}}/k_{\mathrm{D}})_{\max}{}^{4n(1-n)} \qquad (25)$$

It satisfies the expectation that $k_{\mathrm{H}}/k_{\mathrm{D}}$ is a maximum when $n = {}^1/_2$, the symmetric case. Further, $k_{\mathrm{H}}/k_{\mathrm{D}} \cong 1$when the TS is solely in the reactants' valley ($n = 0$) or in the products' valley ($n = 1$) of the potential-energy surfaces. (The deuterium effect on $\Delta G°$ is typically small.) Equation 25 applies both to model A, using eq 8, and to model B, using eq 13, for n near ${}^1/_2$, but has not been explicitly tested.

Instead, if we define κ as the ratio of tunneling to non-tunneling rate constants $\kappa = k_{\mathrm{rate}}^{\mathrm{tunn}}/k_{\mathrm{rate}}^{\mathrm{notunn}}$, one expects κ to equal 1 when the TS is solely in the reactants' valley or solely in the products' valley and to be a maximum at $n = {}^1/_2$. As noted in Part 1, an expression somewhat more accurate than eq 25 is

$$\kappa = (\kappa_{\mathrm{sym}})^{4n(1-n)} \qquad (26)$$

and so $\kappa = \kappa_{\mathrm{sym}}$ when $n = {}^1/_2$, and κ_{sym} is given in Part I. Equations 22−26, like eqs 28−33 given later, have the right limits but their detailed functional form has not been derived by a theoretical argument and so may well be replaced by more accurate descriptions, current and future. The expressions are suggested for concreteness for qualitative application to experiment or computations.

^{13}C Kinetic Isotope Effect. As discussed in Part I and ref 25 the predicted deuterium KIE decreases with increasing asymmetry but the ^{12}C/^{13}C KIE increases, at least initially: In an asymmetrical system the heavy atom coordinate R becomes a more dominant component of the tunneling reaction coordinate (Figure 4) than at $n = {}^1/_2$ and so the ^{13}C KIE is expected to increase and the deuterium KIE is expected to decrease with increasingly driving force. A possible functional form[9] for the ^{13}C KIE is

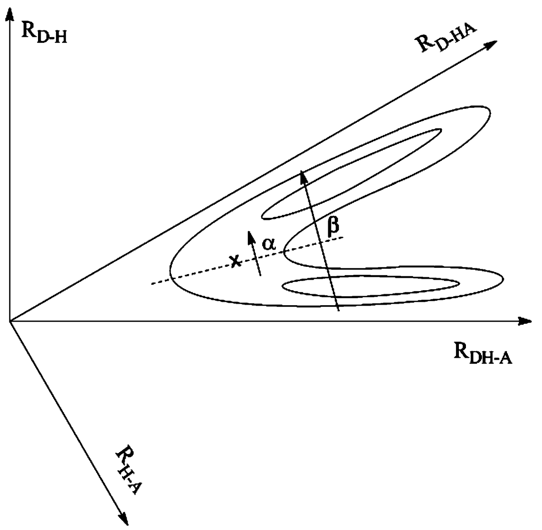

Figure 2. Schematic potential-energy surface for the reaction AH + B → A + HB, using mass-weighted coordinates. X denotes the saddle-point and the α and β paths are indicated. Polar coordinates (R,θ) are introduced, here and in Figures 3 and 4, to define points and paths (cf. refs 22, 25).

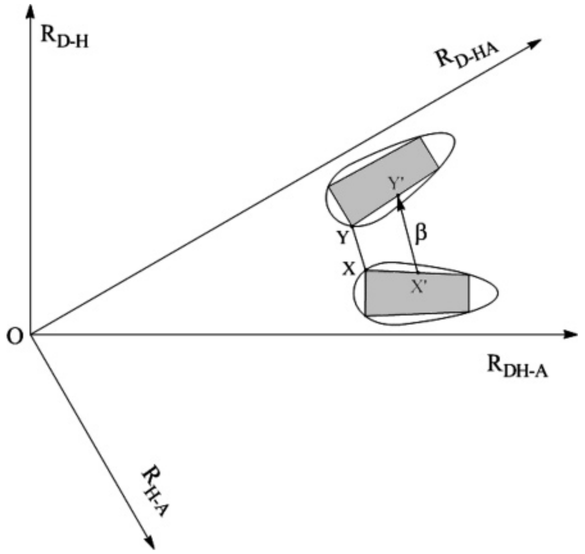

Figure 3. Diagram showing the space swept out by a classical mechanical trajectory in the reactants' well and by one in the products' well and showing tunneling on a β path from point X′ on the boundary of the reactants' distorted rectangle to point Y′ on the boundary of the products' distorted rectangle, including the nearest points X′ = X and Y′ = Y at the corners of the distorted rectangles (cf. refs 22, 25).

$$k_{12}/k_{13} = (k_{12}/k_{13})_{\max}{}^{(1-2n)^2 f} \qquad (27)$$

where n is the n in the TS and $f = 1$ or $n(1-n)$ depending on whether the ^{13}C KIE is a minimum only at $n = {}^1/_2$ or also at $n = 0$ and $n = 1$. The expression in eqs 25−27 are trial expressions.

Specific Results. *Specific Results for Models A and B.* For small $\Delta E^{\circ}_{\mathrm{DHA}}/\lambda_{\mathrm{i}}$ eq 25 yields

$$k_{\mathrm{H}}/k_{\mathrm{D}} \cong ((k_{\mathrm{H}}/k_{\mathrm{D}})_{\max})^{1-(2\Delta E^{\circ}_{\mathrm{DHA}}(R)\ln 2/\lambda_{\mathrm{i}})^2} \qquad \text{(model A)} \quad (28)$$

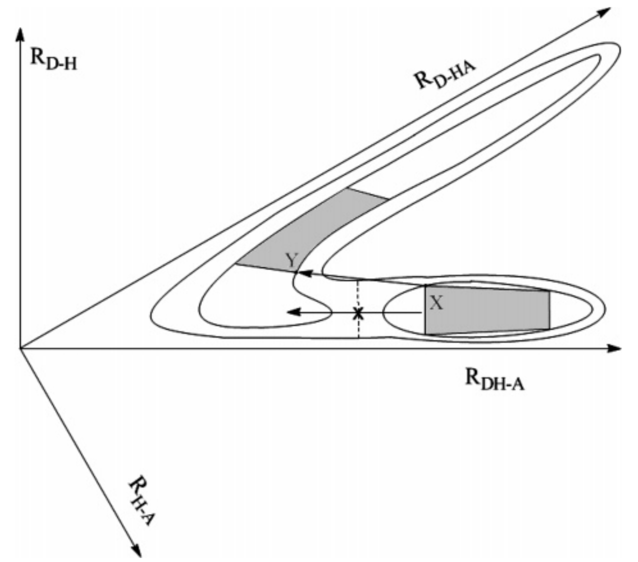

Figure 4. Diagram similar to Figure 3 but for a very downhill reaction, ($\Delta G_R^\circ \ll 0$) (cf. ref 25).

An example of the asymmetric case in this model, namely when $\Delta E_{\mathrm{DHA}}^\circ \neq 0$, is seen in Figure 4, where a tunneling path is also indicated. From eq 22 we similarly obtain

$$\kappa \cong (\kappa_{\max})^{1-(2\Delta E_{\mathrm{DHA}}^\circ(R)\ \ln 2/\lambda_i)^2} \qquad \text{(model A)} \qquad (29)$$

From eq 27 for the ^{13}C isotope effect, using eqs 9 and 26, we have for small $\Delta E_{\mathrm{DHA}}^\circ/\lambda_i$

$$k_{12}/k_{13} \cong [(k_{12}/k_{13})_{\max}]^{-(2\Delta E_{\mathrm{DHA}}^\circ(R)\ \ln 2/\lambda_i)^2 f} \qquad \text{(model A)} \qquad (30)$$

Comparing eqs 9 and 16 that determine *n* for models A and B, respectively, we see that the maximum deuterium KIE for model B occurs at $\Delta G^\circ(R) = 0$, while for model A it occurs at $\Delta E_{\mathrm{DHA}}^\circ = 0$. Instead of eqs 28−30, we now have for small $\Delta G^\circ(R)/\Lambda_2$ in model B,

$$k_H/k_D \cong (k_H/k_D)_{\max}{}^{1-[\Delta G^\circ(R)/\Lambda_2]^2} \qquad \text{(model B)} \qquad (31)$$

$$\kappa \cong (\kappa_{\max})^{1-[\Delta G^\circ(R)/\Lambda_2]^2} \qquad \text{(model B)} \qquad (32)$$

and

$$k_{12}/k_{13} \cong (k_{12}/k_{13})_{\max}{}^{-[\Delta G^\circ(R)/\Lambda_2]^2 f} \qquad \text{(model B)} \qquad (33)$$

5. Experimental Data and Computations

General comments. We recall for comparison the type of data available for electron-transfer reactions in solution and, in part, available for H-type transfers in solution and see how that compares with what is available for enzyme-catalyzed H$^+$, H$^-$, and H$^\bullet$ transfers. For electron transfers the wide range of experimental data includes reaction rates, pre-exponential factors, activation energies, effect of standard free energy of reaction ΔG° on reaction rates, including a predicted inverted effect when $\lambda \ll -\Delta G^\circ$, a relation between rate constants of “cross-reactions” to those of the relevant self-exchange reactions, the effect of solvent, a relation between the ET rate in solution and the corresponding ET reaction rate at an electrode, the effect of the electrode−solution potential on the ET rate, and the relation between thermal ETs and charge-transfer spectra. The predictions and experiments were discussed in Marcus and Sutin,[15] for example. The dependence of ΔG^* on ΔG° in a series of ET reactions is studied with a series of related reactants in which one of the reactants is systematically varied at more or less fixed λ. In some cases using self-exchange data it has been possible to correct for changes in λ, usually via testing the “cross-relation”, for example, ref 20 for electron transfers and ref 23 for methyl-radical transfers. The dependence of the electrochemical rate on the driving force at electrodes is studied by varying the electrode−solution potential difference using a single reactant.

In electron transfers in solution there are thereby many tests of theory and, in particular, of predicted correlations. To a more limited extent it is also the case for ET in proteins and for H$^+$, H$^-$, and H$^\bullet$ transfers in solution. However, such tests are not as accessible for enzymatic H-transfers, as noted earlier. Again, while plots of the experimental ΔG^* versus ΔG° or versus some other free-energy property are common in the literature for chemical reactions in general (“linear free-energy relationships”), the range of ΔG°s over which an H-transfer step in enzymes can be studied is more limited: Prior to and following the H transfer there is a binding of the reactants (substrate and cofactor) or products to the enzyme. The “commitments” (involving the two equilibrium constants) need to be measured or calculated in order to obtain the ΔG^* and ΔG° for the H-transfer step from the experimental data. When the enzyme is saturated with a reactant, there is no need to know the strength of its binding to the enzyme to calculate the ΔG° for the H-transfer step, but to determine ΔG° it is still necessary to know the commitment of the product.

Discussions of enzymes include topics such as how enzymes catalytic properties, $\Delta H^\ddagger$ and $\Delta S^\ddagger$, can differ in the physiological and nonphysiological regimes, on the $\Delta H^\ddagger$ and $\Delta S^\ddagger$ for different transfers, the effect of temperature on the deuterium KIE, the effect on the rate of varying the substrate or cofactor, as well as the effect of pressure on the reaction rates. We consider these topics in the following sections.

A nice example of detailed measurements of the various reaction steps in a hydride transfer is seen in the studies of hydride transfer in dihydrofolate reductase by Benkovic and coworkers for a single substrate and cofactor.[1] When the binding constants can be determined, or otherwise overcome, and when the binding steps are not rate-limiting, both the ΔG^* and ΔG° of the H-transfer step can be measured. There is also another potential complication mentioned earlier in that the “H-transfer step” may involve multiple reaction steps[18] that have to be analyzed separately.

Effect of ΔG°. Examples of experiments or computations on the effect of varying ΔG° by varying the substrate or cofactor in enzymes include experiments by Scharschmidt et al.,[27] Brinkley and Roth,[28] Mihai et al.,[29] Hollfelder and Herschlag,[30] and computations by Bjelic and Aqvist,[31] Schultz and Warshel,[18] and Schweins et al.[32,33] There are also examples where ΔG° is varied by site-directed mutagenesis.[34] The mutagenesis also appears to change λ_o,[8] so complicating the interpretation of the slope of a ΔG^* versus ΔG° plot. We return to this point later. An early study of the YADH catalyzed oxidation of aromatic alcohols was made by Klinman,[35] and a correlation was made with electronic substitution effects. If the ΔG°s are available for the H-transfer step, it would be interesting to explore the correlation of the reaction rates with ΔG°.

In the study of Scharschmidt et al.[27] in liver and yeast alcohol dehydrogenase (LADH and YADH) the NAD nucleotide substrate for LADH was changed to acetyl pyridine−NAD and a similar change was made for YADH, the variation in ΔG°

being 0.062 V. This variation in ΔG° is too small to construct a ΔG^{*} versus ΔG° plot for the reaction step within the complex and a much larger variation of ΔG° is needed. However, the direction of the effect on the rate was the expected one, and there was also the expected effect on the KIEs, discussed later.

In a study of the hydride abstraction by glucose oxidase from flavin cofactors, the ΔG° was varied from -0.5 to -6 kcal/mol (values measured in solution, however), by varying the cofactor.[28] The slope for the bimolecular rate constant $k_{\rm bi}$, $-RT \ln k_{\rm bi}$ vesus ΔG°, was 0.59. The theoretically expected value for a one-step reaction is $\sim$0.5 when $\Delta G^{\circ}(R) = 0$. When the ΔG° for the H-transfer step (termed the intrinsic ΔG°) was estimated from a subset of those data, namely, over a more limited range of only 2 kcal/mol by shifting the overall ΔG° by 2 kcal/mol, the slope for the first-order rate constant was 0.74 instead of $\sim$0.5. Again, experiments over a broader range of intrinsic ΔG°s are desirable. A value of λ was estimated[28] to be $\sim$70 kcal/mol using an equation related to eq 6, but involving the overall λ ($= \lambda_{\rm i} + \lambda_{\rm o}$). These authors also estimated a $\Delta S^{*} \cong 10$ cal mol^{-1} deg^{-1} from the temperature coefficient of the first-order rate constant for the bound complex. Knowledge of ΔS° for this reaction step could assist in understanding ΔS^{*}.

Mihai et al.[29] and Hollfelder and Herschlag[30] studied the effect in phosphotase lipase of varying the p$K_{\rm a}$ of a product of the cleavage of a phosphodiester bond of phosphatidyl inositol[29] and of the cleavage of a phosphoryl bond.[30] In these cases there is no ΔG^{*} versus ΔG° plot, since the p$K_{\rm a}$ is related to both the acidic and the anionic form of a substrate product, but it is only indirectly related to the relevant reactant. A ΔG^{*} versus ΔG° plot can be made if the necessary thermodynamic data become available.

In a proton transfer in the dehydration of HCO_3^- catalysis (in the form of $\log k_{\rm rate}$ vs Δp$K_{\rm a}$) by carbonic anhydrase, Silverman[36] found that a plot of ΔG^{*} versus ΔG° reached a limiting value at a relatively low value of the rate constant. If one assumed only a two-state description, this result would imply a very large $w^{\rm r}$, the residual barrier at a quite negative $\Delta G^{\circ}(R)$. However, as discussed by Schutz and Warshel,[18] the introduction of a third state, in the form of a transfer via a possible H_3O^+ intermediate, provides an alternate and more reasonable interpretation of the data.

To determine the magnitude of $w^{\rm r}$ in computations a systematic variation of $\Delta E^{\circ}_{\rm DHA}$ or ΔG° by varying some parameter in the calculated potential-energy surface would yield $w^{\rm r}$ as the residual barrier at very negative $\Delta E^{\circ}_{\rm DHA}$ or ΔG° . In experiments the variation of the substrate or cofactor could, in principle, be used to determine $w^{\rm r}$ as the limiting barrier at very negative driving force, except in multistep reactions. A systematic study of ΔG^{*} versus ΔG° to obtain $w^{\rm r}$ has been made for a model system by Kreevoy et al.,[22] which also contains a plot of the deuterium KIE versus ΔG^{*}, and a comparison is given with experimental results for the reaction in solution. The effect of not assuming a fixed λ is illustrated there. The change of λ appears in the form of a "tightness" parameter τ.

In a computational study of the initial proton-transfer step in this catalytic reaction of carbonic anhydrase[18] the calculated slope of a ΔG^{*} versus ΔG° plot, obtained by changing a parameter in an EVB potential-energy function, was found to be about 0.6, the deviation from 0.5 being primarily due to $\Delta G^{\circ}/\lambda$ not being negligible. In a study of the hydrolysis rate of GTP bound to a guanosine nucleotide binding protein, Schweins et al.[33] found in their computations a slope of 2.1 for a plot of $\log k_{\rm rate}$ versus ΔpK, instead of the expected value of $\sim$0.5. The range of pK corresponded to a small variation in ΔG° of $\sim$0.5 kcal/mol. The abnormal slope was interpreted in terms of an intermediate (three parabolas), as noted earlier. The intrinsic ΔS^{*} was calculated from the dependence of the rates on temperature and found to be very small, about -0.8 cal mol^{-1} deg^{-1}.

In contrast, in the literature of H-transfers in solution, there are both small and large deviations from a simple linear dependence, with a slope of 0.5 of ΔG^{*} versus ΔG°, due to the quadratic terms in the ΔG^{*} versus ΔG° relation. For example, for a hydride transfer between NAD$^+$ analogues, the λ for the transfer from a CH bond is so high that the quadratic correction term is small over the wide ΔG° range studied.[37] In contrast, in the transfer of an H$^+$ between two oxygens, the λ is quite small, as seen for example in the markedly curved $\ln k_{\rm rate}$ versus $\ln K$ (and so ΔG^{*} vs ΔG°) plots of Eigen and co-workers.[38]

An example where the calculated energy changes $\Delta E_{\rm DHA}$ and the free-energy change ΔG^{*} along a reaction coordinate differ markedly is seen by Alhambra et al.[39] in the transfer of a hydride ion from a carbon to nicotinamide adenine dinucleotide NAD$^+$ in liver alcohol dehydrogenase: LADH + NAD$^+$ $\rightarrow$ LAD + NADH. In these computations the intrinsic energy change in $\Delta E^{\circ}_{\rm DHA}(n)$ is very downhill, but the ΔG° for the overall reaction is close to 0, perhaps in part reflecting a large decrease in entropy accompanying the neutralization of charges in this reaction. Judging from the data on differences in bond lengths in the TS,[39] the transition state occurs when $n \approx {}^1/_2$. It appears on this basis that $\Delta G^{\circ}(R)$ rather than $\Delta E^{\circ}_{\rm DHA}(R)$ controls the deuterium KIE. If so, model B is more suited than model A for this system. Further computational study on the KIE examining the effect of varying $\Delta E^{\circ}_{\rm DHA}$ and $\Delta G^{\circ}(R)$, would be useful, together with similar experimental measurements by varying the substrate or the cofactor.

In an earlier computation by Alhambra et al.[40] for an enolase proton-transfer catalysis of a glycerate to a pyruvate, there was a substantial asymmetry in the TS, using the difference in DH and HA distances as a criterion. The $\Delta E^{\circ}_{\rm DHA}$ was again large and the net free energy of reaction $\Delta G^{\circ}(R)$ was again small. It would seem to be a case of model A. A further analysis of the approximations would be useful to see how this situation arose.

Entropic Effects. Data on the relationship of ΔS° and ΔS^{*} seems to be largely absent in the literature perhaps because of difficulties in the experimental access to ΔS°. One requires the measurement of the intrinsic ΔG° at several temperatures. The measurement of ΔS° for the transfer step can be revealing. If a transfer of an H$^-$ is a charge separation, for example, DH + A $\rightarrow$ D$^+$+ HA$^-$, many of the polarizable groups and orientable groups become more oriented toward the charged products, and as a result the ΔS° of the transfer step is expected to be quite negative, or for the reverse case quite positive. A potential example of the former case, depicted in eq 18, occurs in the study of an enolase by Alhambra et al.[40] mentioned earlier. Orientation effects have been discussed by Olsson et al.[6] and references cited therein.

For the hydride transfer from NADH in morphinone reductase[41] a very positive contribution from the protein structural contribution to ΔG° was found. While the latter was not resolved into contributions from ΔS° and ΔH°, it would be interesting to do so. This reaction[41] provides another example of a very negative ΔS^{*} . The formation of two ions in the reaction yielded ΔS^{*} of about -25 cal mol^{-1} deg^{-1}. Presumably, for the same reason there is also a substantial negative ΔV^{*}, seen in the accelerating effect of pressure on the transfer reaction.[41] Individual measurements of ΔS° and ΔV° would be of interest for a detailed analysis.

In contrast, when the transfer is a charge shift reaction, as in $DH^- + A \rightarrow D + H^- A$, the corresponding contribution to $\Delta S°$ is expected to be relatively small when DH^- and HA^- polarize the protein charges and dipoles to approximately the same extent. With these features in mind the $\Delta S°$ from the experimental data can be revealing. When comparing corresponding $H^+$and H^- transfers in solution and enzymes two major differences in $\Delta S°$ of the transfer step are presumably the differences in "solvation" and mutual orientation of the reactants.

Entropic effects were also the focus of an article in the homolysis dissociation of a carbon−cobalt bond of adenosylcobalamine by ribonucleoside triphosphate reductase.[42] Detailed equilibrium constants in the reaction sequence were evaluated. The ΔS^* and $\Delta S°$ of the dissociative step were found to be $+96 \pm 12$ and $+70 \pm 17$ cal mol^{-1} deg^{-1}, respectively, and the ΔH^* and $\Delta H°$ were 46 ± 7 and 20 ± 8 kcal/mol, respectively. The corresponding ΔG^* and $\Delta G°$ were thereby ca. 16 and ca. −2 kcal/mol. This reaction appears to be another example that has $\Delta G° \approx 0$ owing to a large compensation in $\Delta H°$ and $T\Delta S°$. Depending on the details, the positive ΔS^* and $\Delta S°$ frequently accompany a dissociation. It remains to be assessed how much is due to the conversion of Co(III) to Co(II) on the transfer of an $H^\bullet$ from −SH. Entropic effects in water and in a ribosome have been calculated and compared with each other and would be relevant in discussions of effects in water, as contrasted with proteins.[43]

A puzzling experimental result for $\Delta S^\ddagger$ is the very large negative value (−56 cal mol^{-1} deg^{-1}) seen in the $H^\bullet$ transfer in soybean lipoxygenase.[44] The tunneling factor of 80 only accounts for −9 cal mol^{-1} deg^{-1}. It appears to be due, at least in part, to an electronically nonadiabatic−vibrationally nonadiabatic H^+ transfer.[17,26]

In the case of a thermophilic alcohol dehydrogenase for a hydride transfer,[45] the activation entropy $\Delta S^\ddagger$ was −2.6 cal mol^{-1} deg^{-1} and the $\Delta H^\ddagger$ was 14.5 kcal/mol in the physiological temperature region, whereas at low temperatures $\Delta S^\ddagger$ equals +17.8 cal mol^{-1} deg^{-1} and $\Delta H^\ddagger$ equals 21.2 kcal/mol.

Protein Flexibility. The results for the above thermophilic alcohol dehydrogenase were interpreted as indicating less protein flexibility at low temperatures, the nonphysiological regime.[46] There are two aspects of "flexibility", structural and dynamical: If a protein is more "rigid", its λ_o will be larger. (The analogue to the λ for a chemical bond in ET reactions is $ka^2/2$, so that when λ is larger the equivalent to the bond force constant k is larger, for a given a. That is, the bond is stiffer.) There is also a dynamical aspect: a less flexible protein may have a slower coordinate for the protein motion, the abscissa q_o in Figure 1. When there is a slow diffusive motion toward the TS the population for that slow coordinate near the TS is less than its equilibrium value, since it is not replenished fast enough to compensate for its loss by reaction. To reduce the travel time along that coordinate q_o in Figure 1, the reacting system will utilize q_i more and cross the transition state, the dotted line in Figure 1, to the left of the lowest point on that line.

Thus, because of a slow motion of the protein it can bypass the lowest region of the TS, and its activation energy is higher than it would be otherwise. There is thus a compromise between not proceeding too far along q_o and not going over too high an energy barrier. An example of a treatment with a slow coordinate in a reaction diffusion problem involving electron transfer is given in Sumi and Marcus.[47] There is a large amount of literature on this topic. A change in the slope of a log k_{rate} versus $1/T$ plot in ref 45 may have its origin in a static or dynamic effect on the protein flexibility.

Mutants. In the study of site-directed mutagenesis of amino acid residues on the reaction rate, residues both near and distal have been replaced. The studies provide structural information on the reorganization. Distal residues may have a significant effect on the rate, as seen in Agarwal et al.,[48] Rajagoplan et al.,[49] and Wang et al.[50] and using NMR information on the protein flexibility and fluctuations, by Schnell et al.[51] It has been studied theoretically by Rod et al.,[52] Warshel et al.,[8] and Agarwal and co-workers.[48]

The study of the effect of mutations on the reaction rate includes the hydride transfer in dihydrofolate reductase by Rajagoplan et al.[49] It was found that the intrinsic rate constant for different mutants varied considerably. It was also concluded that the thermodynamics of the reaction varied much less than the rate.

In the case of a double mutation in the hydride transfer in dihydrofolate reductase, the rate was reduced from that of the wild type by a factor of 7000.[50] The adverse change in $T\Delta S^\ddagger$ for the double mutant gave the largest contribution to the decreased reaction rate ($-T\Delta S^\ddagger$ was larger by 3.9 kcal/mol), while $\Delta H^\ddagger$ was a little more favorable ($\Delta H^\ddagger$ was smaller by 1.6 kcal/mol).[50] This adverse $T\Delta S^\ddagger$ may reflect a less favorable steric arrangement and so reflect an increased w^r in the nonphysiological regime. In theoretical computations that do not separate w^r and λ, the result of the mutation would be interpreted as an effect on λ_o. A higher λ_o implies a more "rigid" protein.

In a study of the effect of mutants of tyrosyl-tRNA synthase, the effect of $\Delta G°$ on ΔG^* (and so the effect on the rate constant) was found to have a slope of ∼1.[34] What remains to be assessed is whether the effect of the mutant is due at least partly to changes in λ instead of only in $\Delta G°$. This topic is considered in the next section. Mutation can affect not only λ and $\Delta G°$, but also the w's. To study the effect of $\Delta G°$ on the reaction rate, complementary information may be extracted by varying the substrate or cofactor rather than the mutant. Nevertheless, examining the effects of mutation has provided new insights on structural and relaxation effects in proteins.

In a computation that contains a "reorganization energy" λ in the formalism, typically EVB-based, the effect of the mutation has been primarily on λ, although $\Delta G°$ was also affected.[8] The EVB calculation tacitly incorporates w^r into the "reorganization" and so does not differentiate between the two factors.

Some comparison of enzyme-catalyzed reactions and the analogous reactions in solution are given in Liang and Klinman[45] and Olsen et al.[53] In one example a large difference in KIE for the two media was found.[45] The dramatic effect of mutations does not have its counterpart for reactions in solution.

Deuterium Kinetic Isotope Effect. Among the studies of the deuterium KIE is that of Wang et al.,[50] who studied two single mutants, a double mutant, and the wild type of dihydrofolate reductase. There was the dramatic effect of a factor of 7000 in the reaction rate mentioned earlier for the double mutant with distal amino acid residues. There was also a substantial dependence of the KIE on the mutation, particularly for the double mutant.[50] The long-range coupling of the reactants region to these residues in the distal loops has been supported by NMR studies.[51]

The results indicate that the reactants in the wild-type enzymes are well situated sterically for a tunneling H-transfer, after a reorganization of the environment occurs, and perhaps $w^r = 0$ for the wild type. Particularly for the double mutant this favorable structural arrangement for reaction is absent, and, in the form of a substantial negative entropy of activation, a large $w^{\rm r}$ is needed to adjust the system to enable an H-transfer (gating) and is such that the H may still tunnel but not the D.

The experimental slope of the KIE in hyperthermophilis dimeric DHFR is small or zero in the physiological temperature domain but fairly large in the nonphysiological domain, namely, at lower T values.[54,55] A bond-length-based computation for a thermophilic DHFR has been made and gave a modest T-dependence but did not show a break in the slope of log KIE versus $1/T$ observed in the experiments.[55] Several possibilities were suggested for the absence of a break in the slope in the computer results, including the possibility of a conformational change.[55]

In a study of Scharschmidt et al.[27] of LADH and YADH the intrinsic deuterium and ^{13}C isotope effects were determined as a function of the substrate. The deuterium isotope effect is expected to increase while the ^{13}C isotope effect is expected to decrease when the reaction becomes more “symmetrical”, that is, when the n in the TS is closer to $^1/_2$. This correlation can be understood in terms of the discussion given in section 4.

Among the numerous studies of the effect of temperature on the deuterium KIE, $k_{\rm H}/k_{\rm D}$, are several examples discussed in this section and a later section on the weak temperature dependence for certain systems. The temperature dependence of the KIE was classified broadly in ref 25. Besides the $k_{\rm H}/k_{\rm D}$, the ratio of the corresponding Arrhenius pre-exponential factors $A_{\rm H}/A_{\rm D}$, and the difference of the H and D activation energies $\Delta E_{\rm act}^{\rm HD}$ are measured.

At sufficiently low temperatures, the reaction occurs by tunneling from the zero-point vibrational state of both H and of D. We note that there can still be large protein reorganization and a large change in DH and HA bond lengths appearing in $\Delta E_{\rm DHA}$, both contributing to the activation free energy and activation energy. In this case $A_{\rm H}/A_{\rm D} \gg 1$, and the activation energy difference, $\Delta E_{\rm a}^{\rm HD} = -k\ {\rm d}\ \ln k_{\rm H}/k_{\rm D}/{\rm d}(1/T)$, is close to zero. At somewhat higher temperatures the reaction for D will be “over the barrier”, but when H still tunnels the $A_{\rm H}/A_{\rm D} \ll 1$ and there is significant $\Delta E_{\rm a}^{\rm HD}$ for $k_{\rm H}/k_{\rm D}$ in favor of $k_{\rm H}$. At still higher temperatures, and hence higher E and smaller R values both H and D systems become the over the barrier type and so $A_{\rm H}/A_{\rm D} \approx 1$ (also somewhat >1 or <1), and any $\Delta E_{\rm a}^{\rm HD}$ for $k_{\rm H}/k_{\rm D}$ is smaller than before and is related to the zero-point energy differences. In ref 25 this behavior was labeled as $H_\beta D_\beta$, $H_\beta D_\alpha$, and $H_\alpha D_\alpha$, respectively, where β denotes tunneling (a β-path in Figure 2) and α denotes an “over the barrier” (α-path in Figure 2).

The three classes while serving as convenient designations can have gradations between them. Parenthetically, we note that in a low barrier reaction, H may have a high enough zero-point energy that it passes over the barrier, while the D isotope with its smaller zero-point energy has to tunnel.[40] In the study of the double mutant[50] on the KIE in DHFR mentioned earlier, the double mutant is $H_\beta D_\alpha$, while in wild type it is $H_\beta D_\beta$, as reflected in the activation energies and pre-exponential factor.

Another study is that of Hay et al. on a hydride transfer from NADH by morphinone reductase.[41] The deuterium KIE was about 4 at 25 C. Both $k_{\rm H}$ and $k_{\rm D}$ depended on temperature such that the KIE, $k_{\rm H}/k_{\rm D}$, was also temperature dependent. The simplest interpretation is that it is a $H_\alpha D_\alpha$ case, and thus the temperature effect on $k_{\rm H}/k_{\rm D}$ is primarily due to the differences in zero-point energy between the TS and the reactants. There was also an accelerating effect on $k_{\rm H}$ and $k_{\rm D}$ individually when the pressure was increased, but the $k_{\rm H}/k_{\rm D}$ decreased somewhat. The pressure effect is consistent with the $H_\alpha D_\alpha$ behavior, since pressure would affect this ratio if the reaction were of the $H_\beta D_\beta$ or $H_\beta D_\alpha$ type, as discussed in ref 25.

Proton-coupled electron transfer in lipoxygenase[4,17,26,56] and in nonenzymatic[57] systems can have very large isotope effects because of the long distance for the H-tunneling. In the latter case the deuterium KIE was 400. The role of electronically adiabatic versus nonadiabatic behavior is discussed by several authors, including those in refs 4, 17, and 26 and refs 57−59.

^{13}C Kinetic Isotope Effect. There is an interesting contrast between the ^{12}C/^{13}C and H/D kinetic isotope effects KIEs when the reaction asymmetry $|\Delta E^\circ_{\rm DHA}(R)|/\lambda_{\rm i}$ or $|\Delta G^\circ(R)/\Lambda_2|$ in an enzymatic reaction is changed, as seen in Scharschmidt et al.[27] For example, for one nucleotide substrate the H/D KIE is 6.5 but the ^{12}C/^{13}C is 1.012, and for another substrate which forms a more asymmetrical system (larger $|\Delta G^\circ|$) the H/D KIE decreased from 6.5 to 4 but the ^{13}C KIE increased from 1.012 to 1.025. This result can be understood in terms of the analysis given in section 4. A recent computation of the ^{13}C KIE for models of enzymatic reactions has been made by Gao and Major.[60]

Pressure Effects. A unique study is described by Northrop and co-workers[61,62] concerning the effect of pressure on both the deuterium and ^{13}C KIEs and may be the only such study for ^{13}C KIE. The effects occur over the same pressure range; both KIEs decreasing toward unity with increasing pressure. These effects were discussed in ref 25 in terms of the effect of pressure increasing the ratio of β (tunneling) to α (nontunneling) paths. It would also be interesting to study the effect of pressure on an enzyme for which $k_{\rm H}/k_D$ is considerably greater than the value of 4.9 in the Northrup study, namely a KIE comparable with the high values reported for some other enzymatic reactions, for example, $k_{\rm H}/k_{\rm D} = 40$ (Banerjee et al.[63]) or $k_{\rm H}/k_{\rm D} = 80$ (Knapp et al.,[44] discussed in Hammes-Schiffer and Watney[64]). The pressure effect depends on how close the reaction is to $n \approx {}^1/_2$. For model B, $n \approx {}^1/_2$ requires $\Delta G^\circ(R) \cong 0$, whereas for model A it requires $\Delta E^\circ_{\rm DHA} \cong 0$. The pressure effect would thus be different for the two models.

Weak or Zero Temperature Dependence of Deuterium . When the enzyme is of the wild type and has its natural substrate and operates at its physiological temperature the T-dependence is zero or weak in examples that have come to my attention.[46,54,65−69] Of the several cases considered in an earlier section, $H_\beta D_\beta$, $H_\beta D_\alpha$, and $H_\alpha D_\alpha$, only the $H_\beta D_\beta$ can have a T-independent KIE, and then only when the work term $w^{\rm r}$ is either small or is about the same for the D and H reactions. Indeed, in examples of thermophilic enzymes, the KIE is T-independent in its natural temperatures range, but outside that range the KIE has an activation energy,[46,54] either reflecting the $H_\beta D_\beta$ becoming $H_\beta D_\alpha$ or due to a difference the work terms for H and D systems.

In a study of the T-dependence for *ec*-dihydrofolate reductase, Sikorski et al.[69] observed zero-temperature dependence for the KIE. In a theoretical treatment for this system, Pu et al.[70] calculated a very small $E_{\rm D} - E_{\rm H}$ in the temperature range 5−45 °C, though larger than the experimental value, as noted earlier. At 45 °C they calculated a KIE of 2.81 without tunneling and of 3.1 with tunneling, as compared with the experimental KIE of 3.58 ± 0.15. The calculated value of 2.81 is far less than the semiclassical value of 6 to 7 that occurs when one vibration of the reactants disappears in forming the TS and so suggests some tightening of other vibration frequencies on approaching the TS. The calculated TS for this reaction does not have $n = {}^1/_2$, as seen in the asymmetric DH and HA distances in the TS.[70]

The magnitude of these *T*-independent KIEs depends on the enzyme. In the case of CH bond cleavage by a flavin reduction in sarcosine oxidase, studied by Harris et al.,[68] it was 7.5. In a study of a CH cleavage with trimethyanine dehydrogenase by Basrani et al.,[67] the KIE for the wild type enzyme was 4.5, for pHs where the active group is anionic and so functional. The KIE for one mutant was *T*-dependent. Another example occurs in AcylCoA desaturase, a CH bond cleavage, for which the KIE was 23.[54] For the wild type soybean lipxygenase, involving a proton coupled electron transfer, Knapp et al.[44] found a KIE of 80 at 24 °C and a ΔE_a^{HD} ($= E_a^D - E_a^H$) of 0.9 kcal/mol. This ΔE_a is small, considering the very large magnitude of the KIE. The large KIE is due to the long tunneling distance in the PCET.

Another example where the KIE is *T*-independent or weakly dependent occurs when the temperature is in the physiological range for the enzymatic activity in the study by Maglia and Allemann[54] of a thermophilic dihydrofolate reductase. At 25−65 °C the ΔE_a^{HD} was small, about 0.6 kcal/mol, whereas below that temperature ΔE_a^{HD} was much larger, about 4.6 kcal/mol. Similar results for a hydride transfer in a thermophilic alcohol dehydrogenase were found by Kohen et al.[46] The KIE was *T*-independent $\Delta E_a^{HD}$was ca. 0.5 ± 0.6 kcal/mol for temperatures in the range 30−65 °C, while in the range 5−30 °C it was 7.8 ± 1.8 kcal/mol. In a study by Agarwal et al. of a complex reaction catalyzed by thymidylate synohase the KIE was 3.9 and *T*-independent.[66]

6. Computations

In the present approximate analysis of the experimental and computational results, there are contribution from the quantum-calculated DHA and from the "reorganization" of the protein. The second contribution includes the change in interactions within the protein, the energy stored up in its polarized charges and dipoles, and the DHA/protein interaction. We consider in the next section the two main approaches and suggest a bridge between them.

EVB Approach. In EVB calculations of enzyme reaction rates[3,8] the H-transfer is treated as a transition from one electronic state (DH,A) to another, (D, HA). In the EVB formalism the vertical energy difference ΔE is usually used as a reaction coordinate in the many dimensional DHA/protein coordinate space. The computational results typically yield a pair of parabolic free-energy curves from which a λ can be extracted from the results. Since a DH stretching and an HA compression both contribute to this calculation, the λ obtained in the fit is the sum, $\lambda_o + \lambda_i$. $\Delta G°$ is also obtained, or fit to the experimental value. Perturbation theory is then used to obtain a modified free-energy curve along the reaction coordinate ΔE, using a distance-dependent off-diagonal matrix element describing the interaction between the two valence bond states, and so yielding ΔG^* for the H-transfer step. In practice, the work term w^r is not studied explicitly, but could be evaluated, as discussed earlier, by changing a substrate or cofactor in a way that $\Delta G°$ is made increasingly negative. Then only a w^r will remain. The w^r can also have origins in nonbonded interactions that later influence the reorientation of the reactants.

It is perhaps not surprising that the free energy of each EVB state is approximately parabolic: the protein part is parabolic for the same reason that a parabola describes the free energy of fluctuations of the solvent in ET reactions. (A fixed charge distribution is assumed for each valence bond state). Again in the DHA bonding contribution, although the potential-energy function of each bond is Morse-like and so is anharmonic, the DH bond becomes less stiff and the incipient HA bond becomes stiffer when the system moves along the reaction coordinate, and so the anharmonic effects on the reorganization may approximately cancel.

Bond-Length Difference Approach. In this principal alternative to the EVB approach the difference of bond lengths DH and HA is typically used as a reaction coordinate instead of the vertical diabatic energy gap ΔE, and variational TS theory is then used to obtain the TS.[2,7] To calculate $\Delta E^*_{DHA}(R)$, the DHA contribution to the reaction barrier, a QM electronic structure method is used.[2,7] From the DHA calculations alone, $\Delta E^*_{DHA}(R)$ and $\Delta E^\circ_{DHA}(R)$ can be obtained. From an approximate fit of eq 3 to the reaction barrier in DHA alone (or the modification contained in eq 34 below), a value of λ_i can be extracted. Considering next the calculation with the protein present, the knowledge of $\Delta G°$ (prot) from $\Delta G°(R) - \Delta E^\circ_{DHA}(R)$ in eq 6 and a fit of ΔG^* to eqs 2, 13, and 15 provides a value of λ_o.

However, it would be instructive to obtain a free-energy profile $\Delta G(q,\text{prot})$ along some reaction coordinate q using the bond-length difference method. The coordinate q can be the bond-length difference for example. The computation contains $\Delta E_{DHA}(q,R)$ from the QM results and $\Delta G(q,R)$ from the QM-MM results (all before nuclear tunneling is introduced). The q can be converted to a bond-order coordinate n', defined in eqs 31 and 32 above, and the difference $\Delta G(q,R) - \Delta E_{DHA}(q,R)$ is initially set equal to $n^2\lambda_o$, from which λ_o can be evaluated. Similarly, from the product's side, the difference $\Delta G(q,R) - \Delta E_{DHA}(q,R)$ can be calculated and compared with $(1 - n)^2\lambda_o$, and λ_o can again be evaluated. The two λ_o values may differ unless the formulation of the QM-MM has been symmetrized, since a symmetrization has been assumed in the present formalism.

The λ_o can also be obtained as in the preceding paragraph and compared with these values. A more accurate approach would be to use as a profile the steepest path to the TS in (q_o,q_i) space.[16] (When the protein is not flexible, and if the flexibility is dynamic rather than static, another approach such as an extension of ref 47 would be needed.) A QM-MM computation could provide information on a merging of the two charge distribution (reactants' and products') that would cause deviations from the parabolic model present in eq 6. The error is less if the H jumps from one valley to another and does not come too close to the $n = {}^1/_2$ region. In the case of a proton-coupled electron transfer, as in lipoxygenase, the H^+-tunneling distance is large and the deuterium KIE is correspondingly large, a factor of 80 at room temperature. In dihydrofolate reductase the modest deuterium KIE of 4 means that there is a shorter tunneling distance, and so in this case it remains to be seen how large the deviations from the two parabolas are.

While eqs 2−6 and 13−15 do not have any singularities for the final values for $\Delta E^*{}_{DHA}(R)$ and $\Delta G^*(R)$, the equations that led to these were designed for a reaction in which DH and A are initially at infinity (bond order of HA is $n = 0$) and in which D and HA are finally at infinity (bond order of HA is $n = 1$). However, at a given R the bound complex does not have n exactly equal to 0 initially and n exactly equal to 1 finally. For the purpose of comparing with any free-energy profile we need a function that refers to the bound complex, initially and finally. One simple possibility, that would have an n somewhat greater than 0 initially and somewhat less than 1 finally is a function that is the same as in eqs 13−15, but with n replaced throughout by n'

$$n' = \frac{n - \epsilon}{1 - \epsilon} \tag{34}$$

The advantage of n' over n is that $n = 0$ only at infinite separation of DH and A, and $n = 1$ at infinite separation of D and HA. However, we do not have infinite separation in the bound DHA complex, and so we use the interval $(\epsilon, 1 - \epsilon)$ for n, instead of (0,1). Since n now varies from ϵ to $1 - \epsilon$ in the bound complex, this n' varies from 0 to 1. The n itself is related to bond length in the newly formed bond as in Part I. However, this modified functional form needs to be checked with quantum mechanical electronic structure calculations. The same final equations, eqs 2−6 and 13−15, are obtained but containing n' instead of n. We no longer have infinite separation for reactants and for products.

One might see, for example, the relation between n and the displacement from a "symmetrized"[11] bond-length distance at R, r_e,

$$r - r_e = \gamma \ln n' \tag{35}$$

where γ is a constant chosen to fit the bond order−bond length results and n' is related to the bond order n of the newly formed bond by eq 34. The BEBO model (bond energy−bond order), of which the present eq 3 is a simplified and symmetrized approximation, was formulated by Johnston.[71] The concept of bond order−bond length has it origins in the work of Pauling.

Several comparisons of the numerical results obtained by the two methods have been made and evaluated for a model system[19] and for more enzymatic systems.[72] In addition to the detailed computations of enzyme catalysis there is also a substantial literature on phenomenological models.[73−76] With a choice of parameters it has been possible to fit the weak T-dependence.[44,77]

7. Concluding Remarks

General Remark on Models A and B. The difference between models A and B is large. The analogue in electron-transfer theory would be for model A to have a ΔG^* that equals $(\lambda_i + \Delta G_i^\circ)^2/4\lambda_i + (\lambda_o + \Delta G_o^\circ)^2/4\lambda_o$, while for model B it would be the usual$(\lambda + \Delta G^\circ)^2/4\lambda$, with $\lambda = \lambda_i + \lambda_o$, and $\Delta G^\circ = \Delta G_i^\circ + \Delta G_o^\circ$. Model B for the enzyme catalysis would appear to be the more reasonable. Although there are no explicit coupling terms introduced between the "i" and "o" contributions, there is a tacit coupling assumed in the formulation that led to eqs 13−15 and to Figure 1. It resembles in this respect a central problem in unimolecular reaction rate theory such as RRK (Rice−Ramsperger−Kassel) or RRKM (Rice−Ramsperger−Kassel−Marcus) theory: the final expression contains the result of sharing of the vibrational energy among the various molecular vibrations, even though anharmonic terms responsible for the sharing do not appear explicitly. There is a statistical assumption in that theory and a similarity in the form of using a single coordinate n in model B. There is a statistical assumption of energy sharing between DHA and the protein, even though detailed couplings may not appear explicitly in eqs 13−15. They occur, or should occur, in the Hamiltonian used in computations. In EVB computations the results for DHA plus protein are frequently represented by a single quadratic expression $(\Delta G^\circ + \lambda)^2/4\lambda$, which is the harmonic form of model B.

General Conclusions. Two models are discussed for treating bond breaking−bond forming and reorganization contributions in an H-transfer step in enzymes, one of them related roughly to protein flexibility. The equations, particularly of model B, have implications for the effects of standard free energy and entropy of reaction on free-energy plots and entropies of activation, respectively, and the weak or zero-dependence of the deuterium KIE in certain enzymes. The deduction of parameters of the models from the numerical computations is discussed, together with the effects of pressure and the effect of changes in ΔG° on deuterium and ^{13}C kinetic isotope effects. Approximations in the equations are noted, together with methods of assessing their validity by analysis of the numerical computations. A method is suggested for extracting λ_o from the approach based on a bond-length difference reaction coordinate, typically on an electronically adiabatic surface. The approach is intended to assess the applicability of the quadratic free-energy expression for the protein reorganization.

Acknowledgment. I am pleased to acknowledge the support of this research by the National Science Foundation and the Office of Naval Research. I would like also to acknowledge the very helpful correspondence and suggestions from colleagues in the field: Jiali Gao, Sharon Hammes-Schiffer, Judith Klinman, Amnon Kohen, Don Truhlar, Arieh Warshel and Prof. Maria Michel-Beyerle and Dr. Yousung Jung. It is a particular pleasure to dedicate this article to Norman Sutin, who pioneered much that developed in the field of electron-transfer reactions, the work serving as a prelude to the present analysis.

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(10) A typo there in eq 37 of ref 9 is corrected in the present eq 28. There is also an inadvertent misstatement in the paragraph following eq 19 in ref 9: R is the separation distance reached by the reactants before the change in chemical bonding involved in $\Delta E^*_{\mathrm{DHA}}(R)$. It is not the separation distance in the TS.

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