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Review

# The role of tunneling in enzyme catalysis of C–H activation

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## Abstract

Recent data from studies of enzyme catalyzed hydrogen transfer reactions implicate a new theoretical context in which to understand C–H activation. This is much closer to the Marcus theory of electron transfer, in that environmental factors influence the probability of effective wave function overlap from donor to acceptor atoms. The larger size of hydrogen and the availability of three isotopes (H, D and T) introduce a dimension to the kinetic analysis that is not available for electron transfer. This concerns the role of gating between donor and acceptor atoms, in particular whether the system in question is able to tune distance between reactants to achieve maximal tunneling efficiency. Analysis of enzyme systems is providing increasing evidence of a role for active site residues in optimizing the inter-nuclear distance for nuclear tunneling. The ease with which this optimization can be perturbed, through site-specific mutagenesis or an alteration in reaction conditions, is also readily apparent from an analysis of the changes in the temperature dependence of hydrogen isotope effects.

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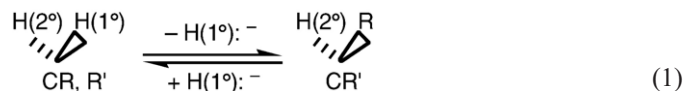
## 1. Introduction

Decades of research on the origins of catalysis in enzyme reactions have shown us that these biological catalysts incorporate simple physical chemical principles to effect enormous rate accelerations. One of the most fundamental reactions carried out by enzymes involves the transfer of hydrogen, either as a proton between an acidic and a basic site comprised of heteroatoms such as oxygen, nitrogen and sulfur or via the direct abstraction of hydrogen from relatively inert carbon centers. The latter reactions, which may occur as a proton, hydride or hydrogen atom transfer, generally involve substrates characterized by large bond dissociation energies and, hence, large inherent barriers for reaction. This feature, when coupled with the capacity of enzymes to create relatively short distances between the hydrogen donor and acceptor that exclude solvent, introduces the possibility of the hydrogen moving through rather than over the reaction barrier. Movement through the barrier (referred to as hydrogen tunneling), though known to occur for solution reactions, had been generally assumed to contribute to a significant degree only at very low

temperatures, i.e., below the temperature niche of ca. 0 to 100 °C occupied by living organisms.

## 2. Early evidence for hydrogen tunneling in enzyme reactions

Beginning in the 1980s, experimental observations began to challenge the semi-classical, transition state view of enzyme catalyzed C–H cleavages. The first inkling that “something was amiss” came from measurements of secondary isotope effects for hydride abstraction from carbon, e.g., Eq. (1):



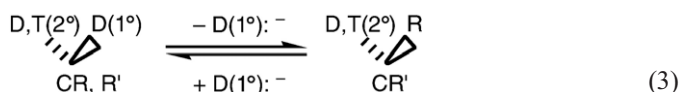
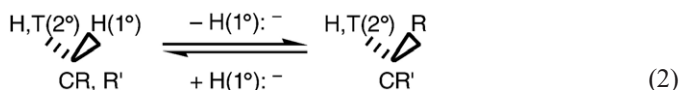
The secondary isotope effect is a measure of the extent to which the reaction rate is reduced when protium is substituted by deuterium or tritium at the non-transferred position and can reflect either a kinetic value, i.e.,  $^{\text{D}}k(2^\circ)$  or an equilibrium value  $^{\text{D}}K(2^\circ)$ . In the context of transition state theory,  $^{\text{D}}k(2^\circ)$  is expected to be less than or equal to  $^{\text{D}}K(2^\circ)$  and independent of the isotope in the primary position, i.e.,  $^{\text{D}}k(2^\circ)$  for C–H transfer =  $^{\text{D}}k(2^\circ)$  for C–D transfer. In fact, both of these predictions were found to break down in several independent systems [1,2], leading to the proposal of a coupling of motion

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at the 1° and 2° positions during the H-transfer process that was accompanied by a tunneling contribution for the hydrogen undergoing transfer, H(1°) [3].

Direct evidence for this behavior emerged in a subsequent study designed to use the isotope tritium (amu of 3) as a frame of reference against which the relative behaviors of H and D could be evaluated [4]. The measurements were, thus, H vs. T at the secondary position during transfer of H, Eq. (2), and D vs. T at the secondary position during transfer of D, Eq. (3):



In the event that the behavior of this system had been contained within the tenets of transition state theory, the secondary kinetic isotope effects measured according to Eqs. (2) and (3) would have been expected to be related by the reduced mass of the respective C–H, C–D and C–T bonds. The experimental data indicated otherwise, with the magnitude of the secondary H/T isotope effect far exceeding that predicted from the measured D/T isotope effect [5]. These studies confirmed the non-classical properties of the enzymatic hydride transfer reaction, inaugurating a subsequent decade and a half of efforts both to detect hydrogen tunneling in enzyme systems and to provide structural and theoretical contexts in which to explain this behavior.

### 3. The tunnel correction

The simplest understanding of the deviant secondary isotope effects outlined above comes from the idea of a tunneling correction. This was introduced quite early in the treatise “The Tunnel in Chemistry” by R. Bell and developed within the

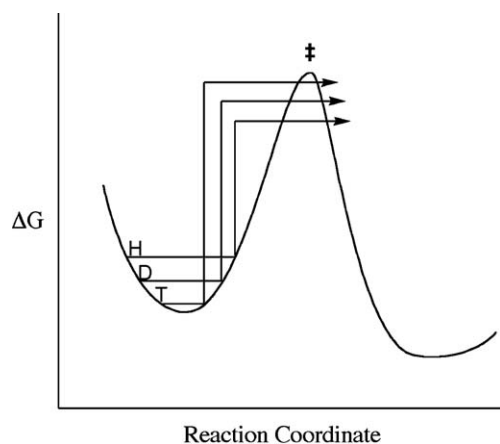


Fig. 1. A cartoon of the Bell tunneling model, emphasizing that a tunneling correction is more pronounced for lighter particles (H>D>T). Reactants have a probability of forming products via tunneling, even when their energy is less than that of the transition-state ( $\ddagger$ ).

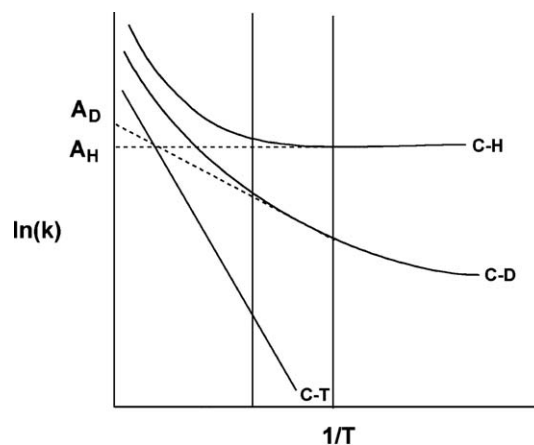


Fig. 2. Illustration of the impact of a tunneling correction on experimentally observed Arrhenius plots. Within a given temperature range, greater curvature of the curve for the lighter isotope gives rise to a smaller value for both the energy of activation and the Arrhenius pre-factor.

framework of a Bell correction [6]. The subsequent emergence of variational transition state theory led to a similar picture, though with considerably more sophisticated theoretical underpinnings [7,8]. The basic aspect of a tunnel correction is visualized in Fig. 1, where the three isotopes of hydrogen are shown moving from reactant to product beneath the barrier, which is expected to become progressively more narrow near its top, leading to a degree of “corner cutting” that is isotope dependent.

The type of behavior shown in Fig. 1 can also explain deuterium kinetic isotope effects that exceed the semi-classical range ( $k_H/k_D \sim 7$ ) [9], as well as the deviant Arrhenius behavior often seen when the transfer of protium and its isotopes is studied as a function of temperature (cf. [10]). The most common observation with regard to the latter is that the degree of curvature in Arrhenius plots is isotope dependent. This leads to differences in the energies of activation for H, D and T, obtained from tangents to experimental Arrhenius curves measured within an accessible temperature range, that deviate from the values expected from the isotopic differences in zero point energy, with  $E_a(\text{H}) \ll E_a(\text{D}) < E_a(\text{T})$ , Fig. 2. A second consequence of such behavior is that intercept values for the Arrhenius pre-factors,  $A_H$ ,  $A_D$  and  $A_T$ , fail to converge toward a single point, as predicted from transition state theory, such that  $A_H/A_D < 1$  and  $A_H/A_T \ll 1$ .

Several investigators have questioned whether the observed deviations, especially those seen in the behavior of secondary isotope effects, could be explained by phenomena that still conform to a semi-classical state view of H-transfer.

Table 1  
Enzymatic examples of non-classical Swain–Schaad relationships for secondary isotope effects

Enzyme	$(k_D/k_T)^{\text{EXP}} = k_H/k_T$	Reference no.
YADH <sup>a</sup>	EXP=10	[5]
HLADH <sup>b</sup> , L57F mutant	EXP=8.5	[34]
<i>B. stearothermophilus</i> ADH <sup>c</sup>	EXP=15	[32]

<sup>a</sup> Yeast alcohol dehydrogenase.

<sup>b</sup> Horse liver alcohol dehydrogenase.

<sup>c</sup> Alcohol dehydrogenase from *B. stearothermophilus*.

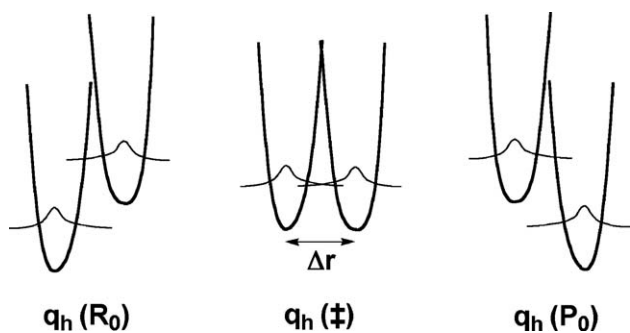


Fig. 3. A multi-dimensional representation of tunneling showing the hydrogen coordinate for the reactant on the left and the product on the right. For wave function overlap to occur, environmental sampling is necessary to achieve transient degeneracy of the reactant and product ground states, together with the optimal distance between the donor and acceptor atoms,  $\Delta r$  (middle drawing). The value of  $\Delta r$  is  $r_0 - r_x$ , cf. Eq. (8).

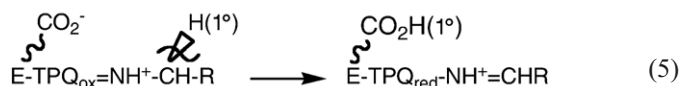
For example, using a term “EXP” to relate a measured secondary H/T to D/T isotope effect, Eq. (4):

$$\{k_D/k_T(2^\circ)\}^{\text{EXP}} = \{k_H/k_T(2^\circ)\} \quad (4)$$

the experimental range for this value can be compared to theoretical upper limits in the absence of tunneling. Though several important investigations have shown that specific conditions can inflate the value of EXP beyond its semi-classical value of 3.36 in the absence of tunneling [11,12], in no instance has it been possible to reproduce the large enzymatically measured values for both  $k_H/k_T$  (ca. 1.3) and EXP (Table 1) without the inclusion of tunneling properties.

#### 4. An alternate view of hydrogen transfer: A model for full tunneling by all isotopes in the bovine serum amine oxidase reaction

Early investigations of the temperature dependences of  $k_H/k_T$  and  $k_D/k_T$  in an enzyme catalyzed reaction were carried out with bovine serum amine oxidase [13], a topa quinone (TPQ)-containing protein that catalyzes the formation of a Schiff base complex between an amine substrate and a protein derived quino-cofactor, followed by proton transfer from this covalent adduct to an active site base (aspartate) [14], Eq. (5):



A major challenge in using Arrhenius parameters to infer that deviations from classical predictions are due to tunneling is to establish conditions under which the C–H bond cleavage is fully rate determining across the experimental temperature range. In the case of bovine serum amine oxidase, the very large size of the  $k_H/k_D$  isotope effect (13.5) at 35 °C indicated both non-classical behavior and a dominance of the measured kinetic parameter by C–H cleavage at this temperature. The further finding that the interrelationship of the primary  $k_H/k_T$  and  $k_D/k_T$  values stayed constant with temperature supported the view that hydrogen

transfer was the dominant step under all conditions. Finally, the analysis of the temperature dependence of  $k_D/k_T$ , in which the rate for hydrogen abstraction had been reduced on average by 13.5-fold relative to  $k_H/k_T$ , removed any ambiguity regarding the non-classical behavior of the hydrogen transfer in this reaction [13].

One of the dominant characteristics of the bovine serum amine oxidase reaction is the large inverse value of  $A_H/A_T$  and  $A_D/A_T$ , originally discussed in the context of a significant tunneling correction. However, these experimental findings caught the attention of several biophysicists at UC Berkeley, who pursued an alternate model for the observed behavior. Taking a lead from electron transfer theory, Bruno and Bialek embarked on an analysis that had the following features [15]: (i) all isotopes of hydrogen were assumed to move by a full tunneling mechanism; (ii) the energy barrier to the reaction was attributed to the motions of the heavier atoms in the environment; and (iii) the change in distance between the hydrogen donor and acceptor that was required for wave function overlap was isotope dependent, with deuterium being transferred from a shorter distance due to its larger mass (amu of 2). In order to simplify their model, the assumption of an isoenergetic reaction was assumed, so that energy terms to transiently achieve this state could be neglected [16]. Remarkably, the experimental data could be very well fit, ushering in a different conceptual basis for our understanding and interpretation of enzyme catalyzed hydrogen transfer reactions.

#### 5. A general approach to C–H activation involving full tunneling of all isotopes

Over the years a fairly extensive literature, emanating initially from a Russian school of theoreticians [16], has addressed the issue of the correct theoretical context for hydrogen transfer. This work has been markedly influenced by the Marcus theories of electron transfer in that barriers to reaction are focused on the environmental reorganizations that are necessary to achieve movement of an electron or hydrogen from the donor to acceptor site (e.g., [17,18]). In the context of enzyme catalyzed C–H abstraction reactions, the conceptual frameworks of Borgis and Hynes [19,20] and Kuznetsov and Ullstrup [21] are particularly notable. In the work of these authors, the multi-dimensional nature of H-transfer is paramount, with separate reaction coordinates being used to represent the physical processes that control tunneling. As illustrated in the left hand profile of Fig. 3, a hydrogen coordinate can be drawn that incorporates the zero point energy for protium and deuterium. In a Marcus type picture, efficient wave function overlap between the donor and acceptor requires degeneracy in the energy levels of reactant and product. Illustrating this property using a reaction that is thermodynamically uphill, the middle profile in Fig. 3 shows the transiently degenerate state that can lead to delocalization of the reactant wave function into the product well.<sup>1</sup> When reaction occurs from the ground state vibrational state for the C–H bond,

<sup>1</sup> Though illustrated for the lowest vibrational level of both reactant and product, wave function overlap may also occur from, for example, an excited vibrational mode of reactant into the zero point vibrational mode of product.

the barrier for this process,  $\Delta G^\ddagger$ , is dependent on two parameters, the reaction driving force  $\Delta G^\circ$  and the reorganization energy,  $\lambda$ , Eq. (6):

$$\Delta G^\ddagger = (\lambda + \Delta G^\circ)2/4\lambda \quad (6)$$

while Eq. (6) derives from electron transfer theory, several important features distinguish electron and hydrogen transfer processes. First, there is the issue of drastically different wavelengths between these particles, such that electron tunneling can occur over very long distances (10–30 Å), whereas hydrogen tunneling is expected to occur over the short distance of 0.6–0.7 Å. This implies a much greater dependence on small changes in distance between the donor and acceptor atoms for hydrogen transfer, such that even small perturbations in distance can impact the probability of hydrogen tunneling very substantially. This is illustrated in the middle profile of Fig. 3 by the term  $\Delta r$ , which introduces a second heavy atom barrier for reaction that derives from the increased van der Waals repulsions that arise as the donor to acceptor distance is reduced. In a simple but physically illuminating picture, the probability of tunneling, is represented by a Franck Condon term, Eq. (7):

$$\text{F. C. term} = \exp^{-m_H\omega_H r_H^2/2\hbar} \quad (7)$$

where  $m_H$ ,  $\omega_H$  and  $r_H$  refer to the mass, frequency and distance traveled for the hydrogen and  $\hbar$  is Planck's constant over  $2\pi$ . Integration of the wave function overlap over a range of possible distances between donor and acceptor, leads to the expression in Eq. (8):

$$(\text{F. C. term})^{\text{gating}} = \int_{r_1}^{r_0} \exp^{-m_H\omega_H r_H^2/2\hbar} \exp^{-(E_x/k_B T)} dX \quad (8)$$

where  $E_x = \frac{1}{2} \hbar \omega_x X^2$  and  $X = r_x \sqrt{m_x \omega_x / \hbar}$ . In this equation,  $m_x$ ,  $\omega_x$ , and  $r_x$  reflect the mass, frequency and distance traversed by the fluctuating barrier;  $k_B$  is the Boltzmann constant. The final probability for tunneling reflects the terms that describe the sampling of different energy states for reactant vs. product and the different distances between reactant and product, Eq. (9) [22,23]:

$$k_{\text{tun}} \alpha (\exp^{-(\lambda + \Delta G^\circ)^2/4\lambda RT}) (\text{F. C. term})^{\text{gating}} \quad (9)$$

A second critical difference between a formulation of tunneling for hydrogen in relation to the electron is the existence of isotopes of the former, H, D and T. This allows us to deconstruct Eq. (9) into components that are temperature dependent from those that are mass dependent, providing unique physical insight into the environmental factors that control the H-tunneling process. First, if we examine the simple F. C. term (without gating) in Eq. (7), we see that this is mass dependent but temperature independent, i.e., it represents a static model of tunneling that is independent of temperature. The F. C. term determines the size of the isotope effects, arising from the differences in the mass of the transferred particles and the initial distance between the donor and acceptor atoms. When the donor and acceptor undergo a close approach, the efficiency of both H and D transfer is high and the measured kinetic isotope effect can

be quite small. Alternatively when the distance between the donor and acceptor becomes large, the smaller wavelength of D (resulting from its larger mass) leads to a reduced wave function overlap for the heavier isotope and the measured kinetic isotope effect can become enormous.

The temperature dependence of Eq. (9) derives from two terms: the Marcus expression, Eq. (6), and the integral that reflects the change in tunneling probability as the distance between the donor and acceptor varies, Eq. (8). Once again, these two terms have different properties, with the Marcus term being largely independent of isotope (as long as the majority of reaction occurs from the ground state vibrational level of the C–H bond), while the integral of tunneling probability with distance is substantially isotope dependent. This latter property comes from the fact that deuterium transfer requires a closer approach between the donor and acceptor atoms, with an attendant increase in its energy of activation.

Thus, there are, fundamentally, three exponentials that contribute to Eq. (9): a mass dependent, temperature independent term, Eq. (7); a temperature dependent and largely mass independent term, Eq. (6); and a term that is both temperature and mass dependent, Eq. (8). The remarkable feature of Eq. (9) is that it allows us to assess which features dominate the measured reaction barrier [21,22]. For example, if the approach between the donor and acceptor atoms can be optimized, very little distance sampling may be required, such that the probability of H and D transfer as a function of temperature is quite similar; this property leads to the prediction of temperature independent isotope effects. Conversely, if the initial distance

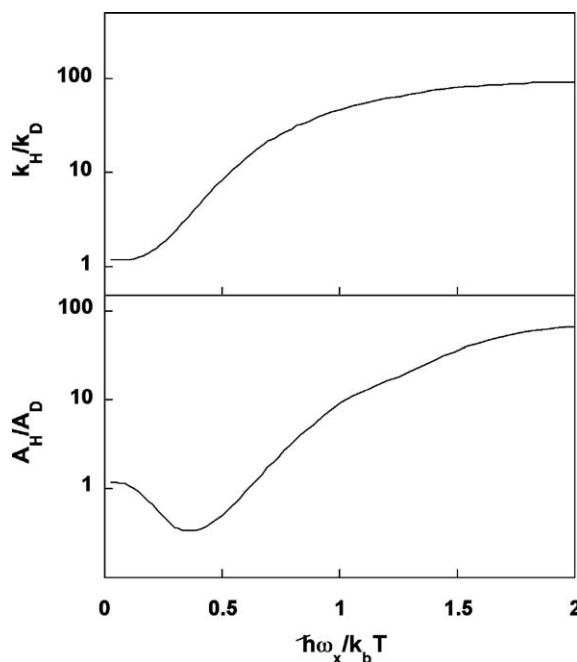


Fig. 4. An illustration of the possible trends in the isotope effect ( $k_H/k_D$ ) and the temperature dependence of the isotope effect ( $A_H/A_D$ ), according to the model for tunneling in Eq. (9). The experimental variables will depend on the initial distance between donor and acceptor atoms, together with the contribution of a gating mode. The latter increases as the potential for gating “softens”, i.e., when  $\hbar\omega_x/k_b T < 1$ .

Table 2  
Enzymatic examples where  $A_{(\text{light})}/A_{(\text{heavy})} < 1$ , from the temperature dependencies of the kinetic isotope effects

Enzyme	$k_{(\text{light})}/k_{(\text{heavy})}$	$A_{(\text{light})}/A_{(\text{heavy})}$	Reference no.
BSAO <sup>a</sup>	(H/T) 35	(H/T) 0.12	[13]
MAO <sup>b</sup>	(H/T) 22	(H/T) 0.13	[35]
GO <sup>c</sup>	(D/T) 2.2	(D/T) 0.57	[36]
GALO <sup>d</sup>	(H/D) 16	(H/D) 0.25	[37]
MMCoMutase <sup>e</sup>	(H/D) 36	(H/D) 0.08	[38]

<sup>a</sup> Bovine serum amine oxidase.

<sup>b</sup> Monoamine oxidase.

<sup>c</sup> Glucose oxidase.

<sup>d</sup> Galactose oxidase.

<sup>e</sup> Methylmalonyl CoA mutase.

between the donor and acceptor is large, there may be a significant need for distance sampling, leading to the prediction of a temperature dependence of the kinetic isotope effect. In fact, Eq. (9) provides an “umbrella” picture of the origin of kinetic isotope effects for H-transfer reactions, leading to the prediction of a wide range of kinetic isotope effects as well as temperature dependences for these isotope effects, illustrated in Fig. 4. This is compatible with the wide ranges of behavior observed for enzyme-catalyzed reactions, Tables 2 and 3.

It is important to point out that there are a number of limitations to Eq. (9), which include, first, the treatment of the H-transfer as a non-adiabatic process; this may be reasonably correct for hydrogen atom transfer reactions but is certainly less correct for the charge transfer that occurs when moving a hydride ion or a proton from donor to acceptor. The model is very simple in that it does not allow for a change in the shape of the hydrogen barrier as a function of distance variation. Further, the distance variation has been treated by assigning a classic harmonic oscillator function to the environment. A number of these features have been addressed in the model of Hammes-Schiffer and co-workers [23], which allows for changes in the barrier shape with changes in distance as well as quantum behavior for the oscillator that controls donor and acceptor distance. In the context of the soybean

Table 3  
Enzymatic examples where  $A_{(\text{light})}/A_{(\text{heavy})} > 1$ , from temperature dependence of the kinetic isotope effects

Enzyme	$k_{(\text{light})}/k_{(\text{heavy})}$	$A_{(\text{light})}/A_{(\text{heavy})}$	Reference no.
SLO <sup>a</sup>	(H/D) 81	(H/D) 18	[21]
Ht-ADH <sup>b</sup>	(H/D) 3.2	(H/D) 2.2	[32]
PHM <sup>c</sup>	(H/D) 10	(H/D) 5.9	[39]
MADH <sup>d</sup>	(H/D) 17	(H/D) 13	[40]
TMADH <sup>e</sup>	(H/D) 4.6	(H/D) 7.8	[41]
SADH <sup>f</sup>	(H/D) 7.3	(H/D) 5.8	[42]
AcCoA Desat. <sup>g</sup>	(H/D) 23	(H/D) 2.2	[43]
DHFR <sup>h</sup>	(H/D) 3.5	(H/D) 4.0	[44]

<sup>a</sup> Soybean lipoxygenase.

<sup>b</sup> High temperature alcohol dehydrogenase.

<sup>c</sup> Peptidylglycine- $\alpha$ -hydroxylating monooxygenase.

<sup>d</sup> Methylamine dehydrogenase.

<sup>e</sup> Trimethylamine dehydrogenase.

<sup>f</sup> Sarcosine dehydrogenase.

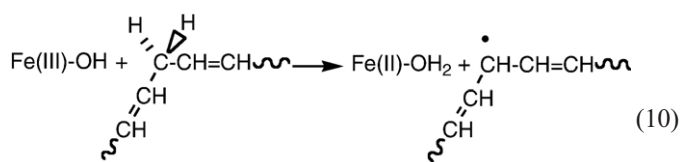
<sup>g</sup> Acyl CoA desaturase.

<sup>h</sup> Dihydrofolate reductase.

lipoxygenase reaction (see below), recent work from this laboratory has addressed the impact of anharmonicity in the C–H well on the predictions of Eq. (9).

## 6. Insight into the role of the environmental reorganization in the reaction catalyzed by soybean lipoxygenase

Though aberrant behavior has been observed in a large number of H-transfer enzyme reactions, the properties of lipoxygenase place this enzyme in a class by itself. The very large size of the experimental isotope effect with wild type enzyme,  $k_{\text{H}}/k_{\text{D}} \cong 80$ , has been verified under a range of conditions [24–27]; further, it has been shown to arise almost exclusively from the transferred ( $1^\circ$ ) hydrogen and without possible complications of interpretation arising from reaction branching or magnetic interactions with the active site iron atom. As illustrated in Eq. (10), the activation of substrate involves a net transfer of a proton and electron to an active site ferric-hydroxide to produce a delocalized radical center and ferrous-water:



Though this system is formally a proton coupled electron transfer, with the proton going to the oxygen and the electron going to the iron center, it is best formalized as a “concerted”

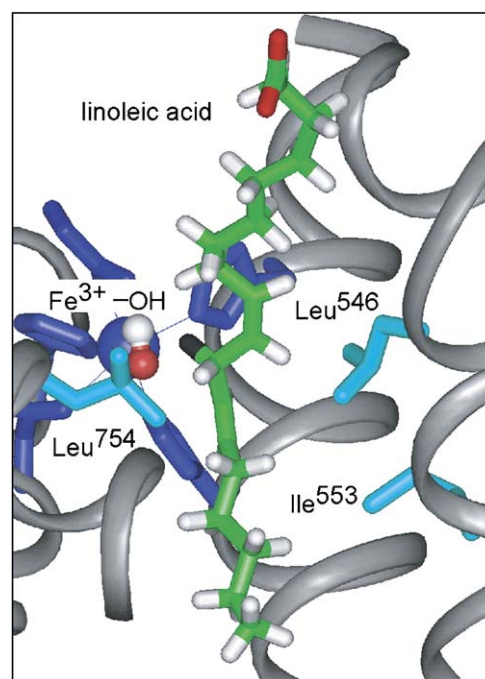


Fig. 5. A picture of the active site of soybean lipoxygenase, from the protein X-ray structure [45], showing a position for linoleic acid derived from an energy minimization [21]. Note the proximity of the reactive C–H of substrate (shown in black) to the residues Leu 754, 546 and Ile 553.

hydrogen atom reaction given the steep uphill energetics for moving either the electron or the proton in the absence of its partner [23].

In addition to the sheer size of the observed deuterium isotope effect, the temperature dependence of protium transfer indicates a very small enthalpy of activation that is only slightly increased upon deuteration. This latter feature produces a weakly temperature dependent isotope effect such that  $A_{\text{H}}/A_{\text{D}}=18$ , i.e., an isotopic Arrhenius pre-factor that is very significantly greater than unity [21].

This can be understood in the context of an active site that is highly optimized with regard to the geometrical placement of the donor and acceptor atoms, such that relatively little distance sampling is needed to achieve efficient wavelength overlap for both deuterium and protium. However, this situation appears to be highly dependent on the protein environment with single point mutations at three separate positions, Leu 546, Leu 754 and Ile 553, increasing the temperature dependence of the isotope effect. Mutations to Ala have been made at positions 546 and 754 (Fig. 5), residues on either side of the reactive carbon of substrate [21], leading to a significant decrease in rate and a reduction in  $A_{\text{H}}/A_{\text{D}}$  to close to unity. In the case of position 553 (Fig. 5), which is a turn away from 754, the side chain has been studied in greater detail via substitution with Val, Ala and Gly [21,28,29]. In each instance, a decrease in the size of the side chain increases the temperature dependence of the isotope effect, with  $A_{\text{H}}/A_{\text{D}}$  becoming as small as 0.027 in the case of Ile 553Gly [28, 29]. X-ray crystallographic studies of the Ile 553 mutant series indicate virtually identical structures at near atomic resolution with the exception of minor differences in side chain rotation of one of the ligands to the active site iron and Ile 754 [29]. These data argue that the origin of the change in the temperature dependence of the isotope effect is not structural. Our interpretation is that alterations in packing density near position 553 impact the configurations available for the C–H of substrate in relation to the ferric-hydroxide. As the bulk of the side chain is decreased and the size of the active site cavity increased, it becomes more and more difficult to achieve the “ideal” configuration of WT-enzyme. Remarkably, the size of the kinetic isotope effect at room temperature remains fairly constant throughout the series of site specific mutants, implying a fairly similar final distance for hydrogen transfer within the wild-type and mutant proteins. The impact of the mutations is, thus, to make it harder for deuterium to reach the critical configuration, leading to the increased  $E_{\text{a}}(\text{D})$  in relation to  $E_{\text{a}}(\text{H})$  and the decreased magnitude of  $A_{\text{H}}/A_{\text{D}}$ .

One very interesting aspect of the mutagenesis studies at position 553 is that the rates of protium transfer are not reduced to a very large extent by the side chain alterations (ca. 5-fold for Ile553Gly [28,29]). This shows how, in some instances, the major contributor to the free energy barrier for protium may come from the Marcus term, Eq. (6) above, providing a possible explanation for the observation of linear relationships between the log of the rate constant and  $\Delta G^{\circ}$  in selected hydrogen transfer reactions (cf. [30,31]). Importantly, the temperature dependence of the isotope effect adds a totally different dimension to the analysis, giving an estimate of the degree to

which the initial distance between the donor and acceptor must be altered prior to H-transfer. This type of analysis is most critical in enzyme reactions, where the protein environment is expected to evolve for optimal function. In fact there is a growing list of native enzymes that display nearly temperature independent kinetic isotope effects (cf. Table 3). Additionally, analogous to SLO, alterations to either the protein (by site specific mutagenesis) or the substrate/reaction conditions which compromise active site configurations have been shown to introduce a temperature dependence to the isotope effects (e.g., [21,32,33]). Results of this nature may present some of the most convincing and clear evidence that enzyme active sites have evolved to enhance the hydrogen tunneling process.

### Acknowledgments

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