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Reduction of Ferric Iron Could Drive Hydrogen Tunneling in Lipoxygenase Catalysis: Implications for Enzymatic and Chemical Mechanisms

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Abstract: Recent kinetic measurements of the oxidation of linoleic acid by soybean lipoxygenase show that the thermal rate constants, $k_H = 280$ (± 12) s^{-1} and $k_D = 5.0$ (± 0.1) s^{-1}, are weakly temperature dependent within the temperature interval 30−50 °C. The primary kinetic k_H/k_D isotope effect is almost temperature independent, and is one order of magnitude larger than expected for a single rate determining isotopically sensitive step. It has therefore been predicted that hydrogen tunneling predominates in this enzyme-catalyzed reaction (Jonsson, T.; Glickman, M. H.; Sun, S.; Klinman, J. P. *J. Am. Chem. Soc.* **1996**, *118*, 10319). Our analysis shows that the lipoxygenase reaction can indeed proceed through a wholly quantum-mechanical pathway. While neither a tunneling correction nor ground-state tunneling of a proton through a one-dimensional potential barrier gives a satisfactory explanation for the isotope effects as measured in the experiment, a dissociation involving a two-step mechanism can explain the experimental observations. The first step is the rate-determining step where a hydrogen species tunnels from linoleic acid to lipoxygenase through a two-dimensional potential barrier. The second step is a relaxation process where an electron is transferred from the metastable intermediate to the Fe^{3+} cofactor in the enzyme active site. This electron transfer causes hydrogen tunneling to be effectively irreversible without introducing a temperature dependence to the reaction. We should note, however, that the two steps are not separable, and cannot be seen as a sequence of independent processes. A consequence of this model is that the tunneling pathways of hydrogen and deuterium are not identical, influencing the magnitude of the measured k_H/k_D isotope effect. This model also serves as an explanation for the role of iron in lipoxygenase, in contrast to the role of iron in many other oxygenases. Iron centers in oxygenases are often necessary for oxygen activation, yet there is no evidence that the iron cofactor in lipoxygenase interacts with molecular oxygen at any stage of the catalytic cycle (Glickman, M. H.; Klinman, J. P. *Biochemistry* **1996**, *35*, 12882). In lipoxygenase, it is possible that reduction of the ferric iron cofactor serves as an electron sink that drives hydrogen tunneling. An electron-gated hydrogen transfer mechanism, such as suggested, could also have relevance to an array of non-enzymatic organometallic reactions.

Introduction[1]

Quantum-mechanical tunneling in hydrogen transfer[2] reactions is a well-characterized phenomenon in physics and chemistry.[3−6] In comparison, chemical reactions in biological systems (with the exception of electron transfer) have been for the most part considered to be adequately described by classical

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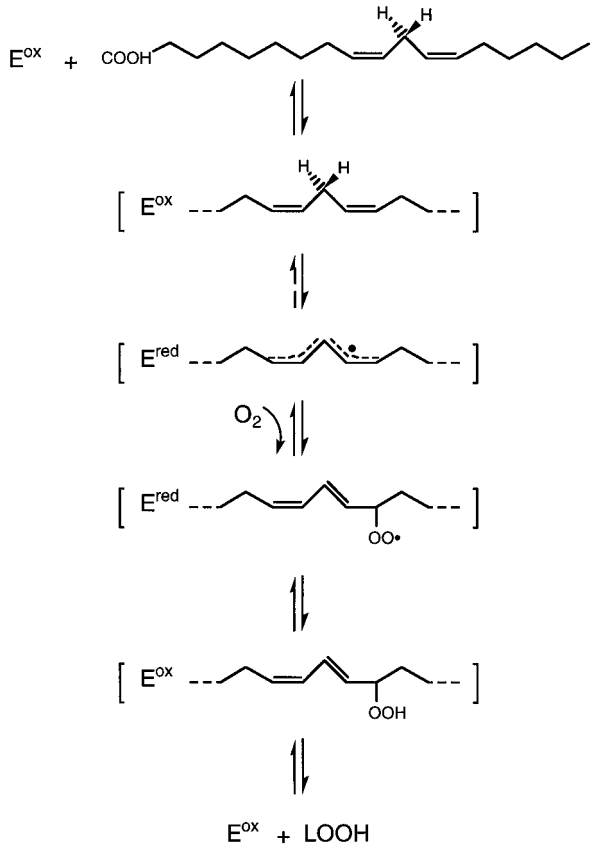
rate theory. The Arrhenius rate equation is a phenomenological description of classical behavior and is exponentially dependent on temperature and the energy of activation (i.e., the height of the barrier but not its width). On the other hand, when taking into account quantum mechanics, the probability that a particle will penetrate a barrier within a measurable time period depends on the height and width of the potential barrier, as well as the reduced mass of the tunneling particle. Within the framework of the semiclassical approach,[4] we can denote the transition probability due to tunneling (T) as exponentially proportional to the square root of the mass of the hydrogen isotope being transferred and to a constant (Θ) that depends on the shape of the potential energy barrier and on the activation energy for dissociation (in our case a C−H bond cleavage), multiplied by a pre-exponential factor, A:

$$T = A \exp(-(m_H)^{1/2}\Theta) \qquad (1)$$

The conventional view of enzyme catalysis, as well as any other catalytic process, is that the height of the potential barrier is reduced (relative to the noncatalyzed reaction), thereby increasing the probability that at a given temperature, the system will have sufficient energy to pass over the barrier.[7,8] In the extreme case, the potential barrier is reduced to such an extent that the hydrogen transfer can be assumed to fit purely classical descriptions. In recent years, however, hydrogen tunneling has been experimentally demonstrated to occur in the hydrogen-transfer steps of at least seven different enzymes: yeast alcohol dehydrogenase,[9,10] horse liver alcohol dehydrogenase,[11] triose-phosphate isomerase,[12] bovine serum amine oxidase (BSAO),[10,13] monoamine oxidase,[14] glucose oxidase (GO),[15] and soybean lipoxygenase-1 (SBL).[16] These experimental observations have usually been explained in terms of a small quantum-mechanical correction to an otherwise classical reaction coordinate, as in the case of the two alcohol dehydrogenases.[17−19] The reactions of GO and BSAO have been suggested to exhibit intermediate degrees of tunneling,[16] and a fully quantum-mechanical model has been developed for the BSAO-catalyzed reaction.[20] Based on experimental observations, the extreme case exhibiting what was empirically predicted to be almost purely quantum-mechanical behavior is the oxidation of linoleic acid by SBL.[16] In this paper, we suggest a mechanism by which an enzyme could bring about a reaction dominated by tunneling.

Scheme 1



Lipoxygenases are a group of non-heme iron dioxygenases that catalyze the oxidation of unsaturated fatty acids to hydroperoxides and are present in numerous eukaryotes (for reviews see refs 21−23). The reaction with linoleic acid (LA), the standard substrate for SBL, preferentially produces 13*S*-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (LOOH) (see Scheme 1). The steady state rate constants for the oxidation of linoleic acid by SBL are $k_H = 280$ (±12) s^{-1} and $k_D = 5.0$ (±0.1) s^{-1} at 32 °C, with unprecedented low activation energies of 1.8 (±0.4) and 2.2 (±0.3) kcal/mol, respectively.[16] It was determined that the C−H bond cleavage is rate limiting above this temperature, yielding a primary k_H/k_D isotope effect of 56 at 32 °C, which is temperature independent within experimental error.[24] The value of this isotope effect is one of the largest ever measured for an enzymatic reaction,[25] and is an order of

(1) List of abbreviations used: SBL; soybean lipoxygenase-1; LA, linoleic acid, or 9(*Z*),12(*Z*)-octadecadienoic acid; LOOH, 13*S*-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid—the product in the lipoxygenase−linoleic acid reaction; BSAO, bovine serum amine oxidase; GO, glucose oxidase; k_H, rate constant for a step (or a reaction) with an unlabeled substrate; k_D, rate constant for a step (or a reaction) with a substrate deuterated in the position to be cleaved; k_H/k_D, the kinetic isotope effect measured by comparing the rate of reaction with protonated substrate to that with deuterated substrate; and 1-D, 2-D, one-dimension, two-dimension, respectively.

(2) The term "hydrogen transfer" is often used loosely to describe reactions involving hydrogen (atom), proton, or hydride particles, even though their chemical properties are remarkably different. Unless specifically mentioned, we will use the term "hydrogen" when referring to a particle that could be any one of the three—hydride, hydrogen atom, or proton.

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magnitude larger than that predicted by semiclassical models for a single rate determining step based on differences in zero-point energies of the two isotopes.[4] A model is required that can explain the fast enzymatic rate of reaction, the low activation energies, and the unusually large temperature-independent primary k_H/k_D isotope effect, and still be consistent with other experimental observations.

The most widely accepted mechanism for lipoxygenase proposes that hydrogen is abstracted from the carbon-11 position on the substrate to yield a delocalized carbon-based radical, which then reacts with dioxygen to produce a peroxyl radical. A proposed kinetic scheme for the lipoxygenase reaction[24,26] is shown in Scheme 1. In this mechanism, hydrogen abstraction from linoleic acid by the active ferric enzyme leads to a carbon-based substrate radical intermediate and a reduced form of SBL. The substrate radical traps molecular dioxygen, leading to a peroxyl radical intermediate. Eventual reoxidation of the enzyme leads to the product peroxide, and regeneration of the enzyme. As the C−H bond cleavage is rate determining under the conditions discussed in this work, we will focus our treatment on this single step—the transfer of a hydrogen from the linoleic acid substrate to the enzyme (eq 2). In this case "A" stands for the substrate linoleic acid bound to the enzyme's active site, "E" is the lipoxygenase enzyme, and "H" symbolizes a hydrogen species.

$$[E + H-A] \rightleftharpoons [E-H + A] \qquad (2)$$

Results

Possible Tunneling Models. Isotope effects are often understood with use of the theory originally described by Bigeleisen and Mayer.[27,28] This successful theory is based on an application of equilibrium statistical mechanics to transition state theory;[29] stable vibrations, in both the ground state and transition state, are treated quantum mechanically, while the reaction coordinate, describing the motion of the transferred hydrogen, is treated classically. Quantum-mechanical corrections to reaction coordinate motion are often added with use of "tunnel corrections" such as the Bell correction.[4] We have attempted to crudely model the large temperature-independent isotope effects seen in lipoxygenase by using a three-center model for the transition state and the Bell correction, examining a variety of different reaction coordinate frequencies and barrier heights. This model seems to be unable to reproduce the experimental trends (data not shown). It is possible to model temperature-independent isotope effects as well as unusually large isotope effects, but not both simultaneously at room temperature. This is consistent with what had been seen previously by Stern and Weston using a somewhat different tunnel correction.[30] In fact, this type of modeling predicts highly temperature-dependent isotope effects as phenomenological manifestations of tunneling. Although these simple calculations cannot rule out the possibility of an explanation for the lipoxygenase data within traditional isotope effect theory, it does suggest that it may be more appropriate to look elsewhere for a satisfactory answer.

We therefore attempted to model the lipoxygenase reaction with simple quantum-mechanical assumptions. Hydrogen tunneling through a symmetrical (or slightly nonsymmetrical) double well potential can be denoted as an equilibrium (such as eq 2 above). The probability of observing the hydrogen bound to linoleic acid, "A", or to the enzyme, "E", oscillates in time and does not give rise to an exponential decay through the potential barrier which is necessary to define a rate constant "*k*", since the tunneling probability in both directions is similar. There are three possible mechanisms by which tunneling back to reactants can be suppressed or eliminated: (1) coupling to other external degrees of freedom such as a thermal bath, (2) extreme nonsymmetry between the two potential wells, and (3) a subsequent fast (temperature-independent) relaxation process. We considered all three options systematically. (1) The first option has been implicated as the driving force in numerous cases of tunneling in both chemical and biological systems.[20,31,32] One should note that coupling to a bath often introduces a temperature sensitivity to the reaction, which is the reason why many reactions exhibiting even large degrees of tunneling are not temperature independent. In the case of lipoxygenase, however, the isotope effect is virtually independent of temperature,[16] and one would not expect the reaction coordinate to be coupled to an external bath of low-frequency modes which would introduce a temperature dependence. However, we cannot absolutely rule out resonant or vibration-enhanced tunneling solely on the fact that there is a temperature dependence too small for us to model. In addition, the overall rates of reaction have a slight temperature dependence though this temperature dependence is similar for both H- and D-labeled substrates. Nevertheless, we feel it is appropriate to seek an explanation in which the hydrogen transfer itself is temperature independent. (2) An exponential decay type behavior can also be obtained for a double-well potential when it is very nonsymmetric; the energy states in the shallow potential well (products) take on a continuum of values into which the hydrogen can spontaneously tunnel. We find this type of explanation highly unlikely to be relevant to most enzymatic reactions. Hydrogen transfer from one carbon atom to another, or even to an oxygen or nitrogen acceptor, would not bring about sufficient nonsymmetry between reactant and product wells to explain the SBL results. Nevertheless, we modeled the SBL reaction assuming a nonsymmetrical double well potential, and found that we could not reproduce both the rates and the large isotope effects measured for the reaction (not shown). (3) In the following paragraphs we will consider the final possibility: a two step process—tunneling followed by a relaxation (damping) mechanism.

Two-Step Tunneling and Relaxation Mechanism. An analysis by Bethe (in order to explain the unusual long lifetime of a hydrogen atom in the 2S orbital when placed in a static field) suggested that in a two-step reaction, the tunneling probability of hydrogen through a barrier can be affected by a subsequent fast relaxation process.[33] More recently, this treatment has been adapted by Lefebvre, Moiseyev, and co-workers to explain the tunneling rates in the automerization of cyclobutadiene,[34] the tunneling rates in the automerization of malonaldehyde,[35] and the rate constants measured for the automer-

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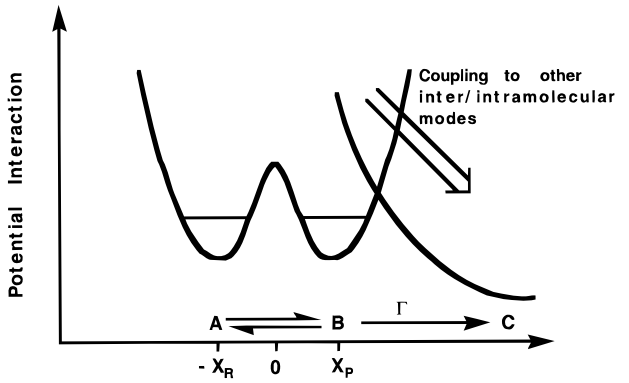

Figure 1. A two-step tunneling and relaxation pathway. Hydrogen is abstracted from reactants (A) by a rate limiting tunneling process through the potential barrier. The short-lived intermediate (B) decays with a rate constant Γ to yield a more stable intermediate along the reaction pathway. Described in detail in the body of the text.

ization of aziridine.[36] In these previous applications of this model, the metastable intermediate was coupled to a bath into which it decayed with a rate constant of Γ. The relaxation, by definition, must be faster than the rate of tunneling, and can be described schematically in Figure 1.

What is the rational for such a two-step mechanism in the SBL reaction? The first step is by definition the rate-limiting isotopically sensitive C−H bond cleavage from linoleic acid. A subsequent relaxation step could be an electron transfer from an unstable enzyme−substrate intermediate to the Fe^{3+} cofactor within the enzyme's active site. Such a proposal can be supported by a number of experimental observations. Free active enzyme is in the ferric state and is then reduced to the ferrous state during the catalytic cycle.[37−39] The C−H bond cleavage precedes dioxygen binding to enzyme,[26] as does reduction of the ferric iron.[16] There is evidence that a carbon-based radical of linoleate can be formed prior to binding of O_2,[40−43] and that this radical is delocalized over one or both of the double bonds flanking carbon-11 from which the hydrogen is abstracted.[25,44,45] The alkyl radical then reacts with oxygen to yield a peroxyl radical.[41,44,46,47] Thus, while the reaction is the equivalent of a hydrogen atom abstraction from substrate, the ferric iron cofactor accepts only an electron—the hydrogen atom abstraction from the linoleic acid substrate separates into a proton and an electron on the enzyme. This is the experimental basis for our "two-step" proposal; the as yet experimentally unanswered question is whether the electron transfer precedes or follows the hydrogen abstraction step. The tunneling model presented in this work will attempt to answer that question. A two-step tunneling and relaxation mechanism for lipoxygenase could be written as a hydrogen abstraction followed by a fast electron transfer to the ferric iron in one of two pathways:

$$[E_{Fe^{3+}} + HA] \rightleftharpoons [E^{H+}_{Fe3+}\ {}^{-}A] \xrightarrow{\Gamma} [E^{H+}_{Fe2+} + {}^{\bullet}A] \qquad (3a)$$

or

$$[E_{Fe^{3+}} + HA] \rightleftharpoons [E^{H}_{Fe3+}\ {}^{\bullet}A] \xrightarrow{\Gamma} [E^{H+}_{Fe2+} + {}^{\bullet}A] \qquad (3b)$$

A hydrogen is cleaved from the linoleic acid substrate (H−A) to yield a short-lived enzyme−substrate intermediate **b**, which then decays with a rate constant of Γ to a more stable intermediate along the reaction pathway ($^{\bullet}$A represents the experimentally detected linoleyl radical, see Scheme 1). We will discuss the nature of intermediate **b** and of this decay in the discussion. However, at this stage we will not make any assumptions as to the chemical mechanism taking place, or as to whether the tunneling step is formally a proton (eq 3a) or hydrogen transfer (eq 3b).

In a two-step classical reaction, as the rate of the second step (Γ in this case) is increased, the rate of disappearance of the reactants is also increased (i.e., the overall rate constant, k_H, increases as Γ is increased). However, when the first equilibrium reaction is a quantum-mechanical tunneling process, an "unintuitive" reversed result is obtained: as Γ becomes larger, the overall rate constant k_H can become smaller. This phenomenon is due to the destruction of the interference effect between the initial tunneling step and the subsequent relaxation. In such a case (see, for example, ref 48, and a simple proof in ref 35) the rate constant is given by eq 4:

$$k_H = \frac{4\nu_H{}^2 T}{\Gamma} \qquad (4)$$

where T is the transition probability through the potential barrier (i.e., tunneling), and Γ is the rate of the subsequent relaxation process. An unintuitive result of eq 4 is that if the rate of Γ is increased, the transition probability of hydrogen transfer from the substrate to the enzyme is suppressed. Once again, it is important to note that eq 4 does not describe a kinetic effect and is valid only when the relaxation step is coupled to the isotopically sensitive step, and is much faster than this step, i.e., $\Gamma \gg k_H$. When modeling the experimental results we will avoid the need to assume the shape and height of the potential barrier. Due to this simplification we benefit in that the ONLY assumption necessary in our treatment is that the reaction dynamics is controlled by a single bound C−H state that is embedded below the top of the potential barrier, and that it is followed by a relaxation.

By substituting eq 1 into eq 4 one obtains:

$$k_H = A\frac{4\nu_H{}^2}{\Gamma}\exp(-(m_H)^{1/2}\Theta_H) \qquad (5)$$

And similarly for deuterium, the rate constant is given by:

$$k_D = A\frac{4\nu_D{}^2}{\Gamma}\exp(-(m_D)^{1/2}\Theta_D) \qquad (6)$$

Tunneling in a One-Dimensional System. Since for a one-dimensional barrier $\Theta_H < \Theta_D$, by choosing $\Theta_H = \Theta_D$ we can approximate a lower limit to the isotope effect (see derivation in footnote 49):

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$$\frac{k_H}{k_D} = \left(\frac{m_D}{m_H}\right)\left(\frac{k_H\Gamma}{4\nu_H^2}\right)^{1-(m_D/m_H)^{1/2}} \quad (7)$$

We can now estimate the isotope effect for the oxidation of linoleic acid by SBL. The C−H bond cleavage, being the rate limiting step, can be assumed to be equal to the overall rate, i.e., $k_H = 280\ s^{-1}$.[16] In our model, the relaxation process Γ is an electron transfer to the Fe^{3+} cofactor from an unstable organic radical produced by the preceding C−H bond cleavage. We may assume the electron transfer is over a distance of 1−2 Å with a rate between 10^8 and 10^{14} Å/s, the latter being the upper limit of the rate of an intramolecular charge transfer.[50] If we take the frequency υ_H to be $10^{14}\ s^{-1}$—as in a typical C−H bond—and the upper limit of $\Gamma = 10^{14}\ s^{-1}$, then the isotopic effect would be expected to be equal to

$$k_H/k_D = 2 \times 10^5 \quad (8)$$

This result is four orders of magnitude larger than the experimentally measured isotope effect. The experimental result, $k_H/k_D = 56$, is obtained only if the frequency of the hydrogen vibration in the reactant well is decreased to $\upsilon_H = 10^9\ s^{-1}$, or the electron transfer rate (Γ) is faster than the speed of light, neither of which are physically relevant cases.

Tunneling through a Multidimensional Potential Energy Surface. In a one-dimensional model there exists only one tunneling pathway that is identical for both hydrogen and deuterium. A two-dimensional potential surface is more accurate in describing the reaction as it allows for the description of additional pathways through which tunneling could occur, and which are not necessarily identical for both isotopes.[51−53] The transition, in a general 2-D plot, is through a bottleneck that is described schematically in Figure 2.

At this stage there is no need to describe the potential energy surface, or to make any assumptions regarding its nature, as the two-step tunneling and relaxation model for the isotope effect includes the multidimensional potential surface in the form of

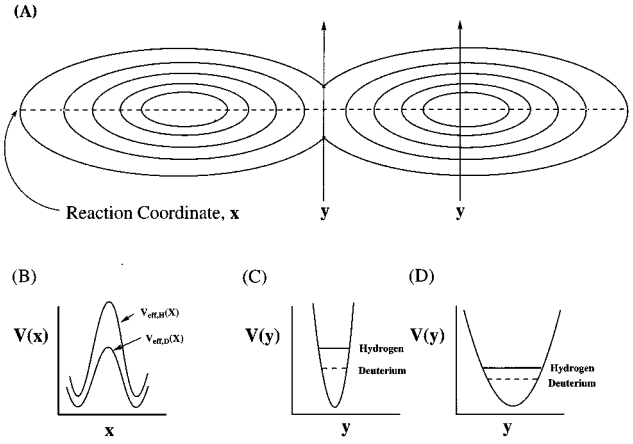

Figure 2. (A) A two-dimensional reaction surface. The reaction coordinate, *x*, describes the motion of the transferred hydrogen. It can also be represented as a double-well potential (B), where the surfaces for hydrogen and deuterium are separated by a zero-point-energy difference. The perpendicular coordinate, *y*, represents an orthogonal mode which is coupled to the reaction coordinate. It can be represented as a stable vibrational potential which has different values along the reaction coordinate as shown in parts C and D. Described in detail in the body of the text.

Θ_H and Θ_D. The expression for the isotope effect is obtained from eq 5−7:

$$\frac{k_H}{k_D} = \left(\frac{m_D}{m_H}\right)\left(\frac{k_H\Gamma}{4\nu_H^2}\right)^{1-(m_D/m_H)^{1/2}(\Theta_D/\Theta_H)} \quad (9)$$

which, not surprisingly, is similar to the one-dimensional expression (eq 7) but takes into account the two-dimensional shape of the barrier in the form of Θ. On the basis of this multidimensional model we can now estimate the conditions that would yield the experimental isotope effect. By using the experimental results $k_H = 280\ s^{-1}$, $k_H/k_D = 56$, and $\upsilon_H = 10^{14}\ s^{-1}$ and a value of $\Gamma = 10^{14}\ s^{-1}$, the isotope effect in eq 9 will be upheld for a ratio of $\Theta_D/\Theta_H = 0.79$.

To summarize, a simple temperature-independent two-step tunneling and relaxation model is consistent with the rates and isotope effects measured for the SBL reaction. A careful look at eq 9 shows that the expression for the isotope effect is insensitive to the rate of relaxation. The theoretical limits of the rate of relaxation are $k_H < \Gamma < c$. Thus the full range of Θ_D/Θ_H varies only from 0.7533 to 0.833 for Γ values of 10^4 to $10^{18}\ s^{-1}$, respectively (and intermediate values of 0.7635 and 07849 for the more likely values of Γ between 10^8 and $10^{13}\ s^{-1}$). The most likely candidate for such a fast relaxation step (coupled to the hydrogen abstraction) is an electron transfer which, as we mentioned above, is experimentally shown to occur during the catalytic cycle. It is important to note that eq 9, which is the concluding result of the two-step model, is not a fit to the experimental results, and does not include any free parameters. The values of k_H, k_H/k_D, m_H, and υ_H are experimentally determined, the ratio Θ_D/Θ_H is fixed by the relationship in eq 9, and while the rate of Γ can vary, it is much faster than the rate of tunneling and does not alter greatly the value of the isotope effect. However, while the value of the isotope effect, based on this model, is insensitive to the rate of relaxation, the overall rate of reaction (k_H as defined in eq 5) is greatly influenced by the value of Γ. This interesting relationship between the presumed electron transfer rate and both the reaction rate and the isotope effect is unique, and could be experimentally tested.

It is also possible to obtain something of a physical picture regarding the meaning of Θ_D/Θ_H. However, in order to obtain

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(49) The derivation of eq 7 is as follows. From the ratio of eqs 5 and 6 one gets:

$$\frac{k_H}{k_D} = \left(\frac{\nu_H}{\nu_D}\right)^2 \exp(-((m_H)^{1/2} - (m_D)^{1/2})\Theta) \quad (a)$$

and by rearranging

$$\frac{k_H}{k_D} = \left(\frac{\nu_H}{\nu_D}\right)^2 \exp\left(-\Theta(m_H)^{1/2}\left(1 - \frac{(m_D)^{1/2}}{(m_H)^{1/2}}\right)\right) \quad (b)$$

or

$$\frac{k_H}{k_D} = \left(\frac{\nu_H}{\nu_D}\right)^2 (\exp(-\Theta(m_H)^{1/2}))^{1-(m_D/m_H)^{1/2}} \quad (c)$$

Using the relationship in eq 5 in the text, we can express the exponent in eq c, $\exp(-\Theta m_H^{1/2})$, in terms of other parameters:

$$\frac{k_H}{k_D} = \left(\frac{\nu_H}{\nu_D}\right)^2\left(\frac{k_H\Gamma}{4\nu_H^2}\right)^{1-(m_D/m_H)^{1/2}} \quad (d)$$

In addition, using the harmonic oscillator relationship, the term $(\nu_H/\nu_\Delta)^2$ is equal to the inverse ratio of the corresponding masses:

$$\frac{k_H}{k_D} = \left(\frac{m_D}{m_H}\right)\left(\frac{k_H\Gamma}{4\nu_H^2}\right)^{1-(m_D/m_H)^{1/2}} \quad (e)$$

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additional information from eq 9 we will need to make several assumptions regarding the shape of the barrier. Θ_H and Θ_D are parameters that depend on the shape of the barrier and are defined simply as an integration:[54]

$$\Theta_{H,D} = \int_{-x_r}^{x_p} (2V_{eff}^{H,D}(x))^{1/2}\, dx \tag{10}$$

(as a note, $-x_r$ and x_p are not identical for the two isotopes; in reality $x_p^D > x_p^H$ and $-x_r^D < -x_r^H$, but this is a small effect that we will ignore). At the same time, for a simple symmetric barrier, we can also assume that the energy of activation is proportional to an integration of the potential along the reaction pathway:[55]

$$E_a \propto \int_{-x_r}^{x_p} V_{eff}(x)\, dx \tag{11}$$

We can now see that the ratio of Θ_D/Θ_H approximates the square root of the ratio of the barrier heights:

$$\frac{\Theta_D}{\Theta_H} \cong \left(\frac{E_a^D}{E_a^H}\right)^{1/2} \tag{12}$$

By using a value of $\Theta_D/\Theta_H = 0.79$ as determined from eq 9,the conclusion from this model is that the "activation energy" for the C−H bond cleavage is about 1.6 times higher than that for the C−D cleavage.

Description of a Possible 2-D Potential Energy Surface. The prediction from the two-step tunneling and relaxation model is that in the SBL reaction, hydrogen tunnels through a higher energy barrier than deuterium. The question then arises, what kind of potential surface could describe such a situation? In the following two paragraphs (eqs 13−20) we describe a possible description of a two-dimensional potential surface; the information obtained from this description can be used to rationalize the concluding tunneling and relaxation expression in eq 9 above, though it was not necessary for deriving it. In thermally driven reactions ("classical" behavior), it is convenient to define the reaction coordinate, *x*, in terms of simultaneous bond breaking (donor C−H bond) and bond making (acceptor C−H bond). This can be represented as a C−H stretching coordinate containing both acceptor and donor C−H stretching vibrations. This is useful because understanding this coordinate can yield information regarding the transition state. In reactions exhibiting quantum-mechanical behavior, however, the reaction coordinate is not necessarily related in any simple fashion to the vibrational modes—the hydrogen tunnels through the barrier and does not necessarily follow the path of lowest energy from reactants to products (see for example ref 54).

In the studied case, therefore, we will not assume the reaction coordinate to be an harmonic oscillator, rather we will first define an adiabatic potential surface, in which the transition of the hydrogen atom is along a reaction coordinate *x*. The effective energy at any given location along this coordinate defines the potential energy surface as a function of the distance between the atoms. At a first approximation, the potential curves for hydrogen and deuterium are similar, since the potential energy is a function of the distance between the donor and acceptor atoms, which is roughly equal for both isotopes. The potential, $Vx(x)$, along the reaction coordinate *x* is a double well potential where $x = 0$ is defined as the transition point between two local minima, $x = -x_r$ and $x = +x_p$, representing the reactant's well and the product's well, respectively. The height of the potential barrier at $x = 0$ is the activation energy (see Figure 2). We reiterate that the reaction coordinate in this tunneling model does not necessarily correspond to the reaction coordinate described by transition state theory. Let us now take into consideration another coordinate, *y*, which is perpendicular to the reaction coordinate, *x*. The perpendicular mode, *y*, could describe for instance a high-frequency hydrogen stretching or bending mode for which the excited states would be insignificantly populated at room temperature, and thus would be expected to contribute only a very slight temperature dependence to the overall reaction. The potential along the *y* coordinate can be described as a harmonic oscillator:

$$V_y = \frac{K}{2} y^2 \tag{13}$$

The zero vibrational potential energy in the *y* direction is $\hbar\omega/2$, where ω is mass dependent since $m\omega^2 \equiv K$. For a first approximation, we will assume *K* is *x* independent, therefore the effective potential along the reaction coordinate (which can be imagined as a bobsled trajectory on a hill, see Figure 2) is defined as follows:

$$V_{eff}^H(x) = V_x(x) + V_y = V_x(x) + \frac{\hbar}{2}\left(\frac{K}{m_H}\right)^{1/2} \tag{14}$$

whereas,

$$V_{eff}^D(x) = V_x(x) + V_y = V_x(x) + \frac{\hbar}{2}\left(\frac{K}{m_D}\right)^{1/2} \tag{15}$$

It is clear that:

$$V_{eff}^H(x) > V_{eff}^D(x) \tag{16}$$

In this case the effective potential is shifted by a constant (relative to the 1-D scenario) at any given location along the *x* axis. Since V_y is a constant, the activation energy for hydrogen or for deuterium remains unchanged from that described by a 1-D barrier, which does not include the V_y parameter. This conclusion can be summarized by eq 17:

$$E_H = V_{eff}^H(x{=}0) - V_{eff}^H(x{=}{-}x_r) = V_{eff}^D(x{=}0) - V_{eff}^D(x{=}{-}x_r) = E_D \tag{17}$$

In reality, however, *K* in eqs 13−15 is a function of *x* and should be notated as $K(x)$, thus introducing (parametric) coupling between the *x* and *y* modes such that the width of the reaction pathway can vary with location along the reaction coordinate, *x*. Depending on the nature of this coupling, the result can deviate from the 1-D model in opposite directions. Provided the saddle point is "tighter" than the reactant well (has a larger force constant in the transverse mode), $K(x)$ would be maximal at $x = 0$ and minimal in the reactant and product regions. In such a case:

$$V_{eff}^{H,D}(x{=}0) - V_x(x{=}0) > V_{eff}^{H,D}(x{=}{-}x_r) - V_x(x{=}{-}x_r) \tag{18}$$

In this scenario, the energy barrier for hydrogen is increased to a greater extent than for deuterium, due to the fact that in the transition point (where the force constant is larger), the zero-point vibrational energy of hydrogen is higher than that of deuterium in the reactant well. Therefore:

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(55) The area of a triangle is linearly proportional to its height, therefore for a symmetric 1-dimensional barrier the energy of activation would be proportional to an integration of the potential over the reaction pathway. In this case, the ratio of energies of activation for H and D would be given by the ratio of the respective integrated potentials, with other constants canceling out.

$$V_{\mathrm{eff}}^{\mathrm{H}}(x=0) - V_{\mathrm{eff}}^{\mathrm{H}}(x=-x_{\mathrm{r}}) > V_{\mathrm{eff}}^{\mathrm{D}}(x=0) - V_{\mathrm{eff}}^{\mathrm{D}}(x=-x_{\mathrm{r}}) \quad (19)$$

or in other words,

$$E_{\mathrm{a}}^{\mathrm{H}} > E_{\mathrm{a}}^{\mathrm{D}} \quad (20)$$

In this case the isotopic effect will be smaller than what was obtained from the one-dimensional potential calculations shown in eq 7 above, due to the fact that tunneling of hydrogen is suppressed to a greater degree than that of deuterium.[56] This conclusion satisfies the relationship in eq 12, which independently rationalized that for a two-step tunneling and relaxation model, hydrogen would tunnel through a higher barrier than deuterium. To summarize, the experimental results can be explained by using a two-dimensional potential surface, in which the *y* coordinate is such that the force constant obtains a maximal value at the transition point.

Discussion

Our primary conclusion is that a purely quantum mechanical treatment of the hydrogen-transfer step can explain the large, close to temperature independent, isotope effects measured for the lipoxygenase reaction. Neither a tunnel correction to a semiclassical rate of reaction nor ground-state tunneling through a static one-dimensional barrier can give in our hands a satisfactory explanation for the behavior seen in the lipoxygenase reaction. Interestingly, a similar conclusion was reached by Bruno and Bialek while trying to model the isotope effects measured for BSAO.[20] Secondly, we show a mechanism by which tunneling can be achieved, and favored in the forward direction, without introducing a temperature dependence to the hydrogen transfer step itself, as would be the case in a vibration-assisted tunneling mechanism (such as proposed, for instance, for the BSAO and GO reactions). We admittedly ignore the possibility of several temperature-dependent components of the rate constant fortuitously canceling each other out and leading to an overall temperature-independent rate. Structure−function studies will either uncover this intrinsic temperature effect or provide further evidence that the reaction is truly temperature independent. Based on the model presented in this article, we propose that the isotopically sensitive C−H bond cleavage is linked to a fast electron reduction of the ferric cofactor making the hydrogen transfer from substrate to enzyme effectively irreversible. Indeed, recent experimental evidence shows the C−H bond cleavage step to be irreversible (while other steps described in Scheme 1 are reversible).[26] We should emphasize, however, that the two steps in this model are coupled, and cannot be seen as a simple sequence of two separate processes.

This model also makes predictions regarding the nature of the potential energy surface of the reaction. In particular, it predicts that hydrogen tunnels along a different pathway—and through a higher energy barrier—than does deuterium. This can be explained by the existence of isotopically-sensitive perpendicular modes, *y*, which have larger force constants in the saddle point through which the hydrogen is tunneling than in the reactant configuration. This situation can be described as a "tight transition state", though once again it should be noted that the transition state as defined in this tunneling theory can be very different than the transition state as defined in classical rate theory. The existence of a "tight transition state" should be verifiable by ab initio calculations of the reaction surface.

Most importantly, this model clarifies the role of the iron cofactor in SBL catalysis. A major role for iron centers in enzymes utilizing dioxygen is to activate molecular oxygen so as to overcome its intrinsic unreactivity toward covalent bonds.[57,58] However, ferric iron can also serve as an electron acceptor in redox reactions. Until now, it has not been clear what role the active site ferric iron plays in SBL catalysis as there is no evidence that the iron cofactor—in either its ferric or ferrous forms—interacts with molecular oxygen at any stage throughout the catalytic cycle.[26,59] The model presented in this paper suggests that the role of the ferric iron cofactor is to serve as an electron sink in order to drive hydrogen tunneling. Such a proposal is in agreement with the unusually high reduction potential[60,61] and the distorted octahedral geometry of the ferric iron.[62] The presented model makes specific experimentally verifiable predictions as to the relationship between the rate of electron transfer and both the rates of reaction and the magnitude of the isotope effects, suggesting that the selection of the transition-metal cofactor plays a non-casual role in catalysis. Decreasing the electron transfer rate would increase the hydrogen transfer rate (and the overall rate of reaction), yet have little influence on the magnitude of the isotope effect! (And vice versa, increasing the electron transfer rate should decrease the rate of reaction, assuming C−H bond cleavage is still rate limiting.) For example, one could examine the enzymatic rate and isotope effect after shifting the redox potential of the ferric iron by alteration of the iron ligands using site-specific mutagenesis. Replacement of the ferric iron with another metal ion, similar in radius and ligand binding capability yet differing in redox potential, might also lead to interesting results. The importance of electron transfer for the overall reaction is already apparent: the ferrous form of SBL is inactive.[37,39]

While the iron cofactor accepts the electron during catalytic turnover, the nature of the enzymatic hydrogen (or proton) acceptor is still puzzling. Active enzyme is in an oxidized ferric state; once treated with linoleic acid the Fe^{3+} is reduced to Fe^{2+}.[37−39] From the crystal structure of SBL there is no obvious candidate for either an active site base or an active site radical, which could accept a hydrogen species from the substrate.[62] However, the crystal structure of SBL has identified a water molecule as a ligand of the ferrous iron in the native form of the enzyme; this ligand points directly into the cavity presumed to be the fatty acid binding site.[62] Furthermore, a hydroxide ligand has been identified as a ligand of the active ferric form of SBL.[63] It was proposed that this ligand could be the active site hydrogen acceptor since it could serve as both a proton and an electron acceptor. It was also shown that this ligand is probably retained and protonated to yield a water molecule in the reduced ferrous complex.[63] A mechanism involving an isotopically sensitive hydrogen transfer followed by a fast electron transfer could then be written as follows:

(56) As a note, in the opposite scenario, whereby $K(x)$ reaches a minimal value at $x = 0$ (i.e., a "loose transition state"), the height of the potential barrier (the "activation energy") is decreased compared to the one-dimensional model described above, and it is decreased to a greater extent for hydrogen than for deuterium. Since even the one-dimensional calculations produced isotope effects larger than the experimental results as seen in eq 7, this regime is obviously not the explanation for the isotope effects observed in the lipoxygenase reaction.

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$$[[Fe^{III}\cdot OH^{-}]^{2+} + H\cdot A] \rightleftharpoons [[Fe^{III}\cdot OH_2]^{3+} + {}^{-}A] \xrightarrow{\Gamma} [[Fe^{II}\cdot OH_2]^{2+} + {}^{\bullet}A] \quad (21)$$

or as:

$$[[Fe^{III}\cdot OH^{-}]^{2+} + H\cdot A] \rightleftharpoons [[Fe^{III}\cdot OH_2{}^{-}]^{2+} + {}^{\bullet}A] \xrightarrow{\Gamma} [[Fe^{II}\cdot OH_2]^{2+} + {}^{\bullet}A] \quad (22)$$

The significance of the relaxation is that it breaks the symmetry on both sides of the barrier through which hydrogen tunnels, by destroying either the hydrogen acceptor (eq 22) or donor (eq 21) therefore the formalism in either eq 22 or eq 21 can be supported by the model presented in this work. It could be argued that a proton transfer to the hydroxide ligand coupled to an electron transfer from the unstable carbanion (eq 21) might make more chemical sense. However, in favor of eq 22, the high spin iron might impart some radical character to the hydroxide ligand; if we think of the $Fe^{III}(OH^{-})$ complex as having a resonance structure with some $Fe^{II}(OH^{\bullet})$ character, this mechanism has appeal in explaining the homolytic C−H bond cleavage. In either scenario the subsequent relaxation, Γ, is nominally an electron transfer between a ligand and a metal core, and thus consistent with the fast relaxation process predicted by the present model. Indeed, there is precedence that the rate-limiting step in a one-electron oxidation can be the formation of a charge transfer complex between an organometallic catalyst and its substrate.[64] To conclude, the theory presented in this paper, in conjunction with the numerous structural and functional experimental observations we have outlined, suggests that an electron-gated hydrogen tunneling from substrate to enzyme is a likely mechanism for the SBL reaction.

In light of the presented model and the other recent results showing that hydrogen tunneling plays a role in enzymatic reactions, it would seem that enzymes can achieve catalysis of a C−H bond through a variety of means. In the case of SBL, both the nature of the iron cofactor and the overall geometry of the active site are crucial in determining the outcome of the catalytic reaction by optimizing the degree of quantum-mechanical tunneling. Other enzymes might maximize the degree of hydrogen tunneling through dynamic fluctuations of the enzyme that bring about a specific thermally "activated conformation" (see for instance refs 15 and 20), and in still others tunneling is a correction to an otherwise thermally driven reaction.[9,17,18] In most enzymes, the reaction proceeds mainly by lowering the energy of the transition state through binding energies (the "classical view"[7,8]).

A two-step tunneling and relaxation model could also have relevance to an array of non-enzymatic organometallic reactions. The mechanism that we propose for SBL catalysis is strikingly similar to certain non-enzymatic oxidation reactions. For instance, a temperature-independent k_H/k_D isotope of 50 at room temperature was measured for alcohol oxidation by ruthenium complexes in solution.[65] The mechanism involves a $[Ru^{IV}{=}O]^{2+}$ catalytic core which accepts a hydride to yield $[Ru^{II}{-}OH]^{+}$. It is possible that this reaction also proceeds in a two-step fashion: an isotopically sensitive hydride transfer to the oxyl ligand, followed by fast reduction of the metal core. It is possible that other reactions, such as those involving a homolytic cleavage with no obvious radical acceptor, could follow a similar mechanism.

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