Experimental Evidence for Extensive Tunneling of Hydrogen in the Lipoxygenase Reaction: Implications for Enzyme Catalysis

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Hydrogen tunneling has been shown to contribute to the reactions catalyzed by alcohol dehydrogenases from yeast[1] and horse liver,[2] bovine serum amine oxidase,[3] and monoamine oxidase B[4] under ambient conditions. We now report evidence for *extensive tunneling of both H and D* in the oxidation of linoleic acid catalyzed by soybean lipoxygenase (SBL).

Recent investigations of steady state kinetic isotope effects in the SBL reaction have indicated the largest deuterium isotope effect reported for a biological system ($k_H/k_D = 48$ at 25 °C).[5] A kinetic picture has emerged in which the rate-limiting step changes from one which is partially limited by substrate binding (at room temperature) to one which is fully rate limited by the C−H bond abstraction (above 32 °C).[6] The temperature dependence of k_{cat} for the steady state oxidation of linoleic acid by soybean lipoxygenase at 32 °C and above is presented in Figure 1. The maximum isotope effect is 56 ± 5 at 32 °C, decreasing slightly with further increases in temperature as anticipated for an isolated C−H bond-cleavage step. The data indicate almost parallel lines with enthalpies of activation of 1.6 and 1.2 kcal/mol for D-LA and H-LA, respectively (Table 1). Extrapolation of the data in Figure 1a to infinite temperature leads to an isotope effect on the Arrhenius prefactor which is enormous ($A_H/A_D = 27 \pm 15$). This behavior, which is in marked contrast to classical behavior that predicts $A_H/A_D \simeq 1$,[7] is a consequence of the fact that the isotope effects are large yet almost independent of temperature.

Unambiguous interpretation of the above data requires the hydrogen transfer to be fully rate limiting within the experimental temperature range. This point has been addressed using pre-steady state conditions of analysis. The most widely accepted mechanism for soybean lipoxygenase invokes hydrogen atom abstraction from the C-11 position of substrate by an active site ferric hydroxide to yield the ferrous form of enzyme and a delocalized radical of linoleic acid (see ref 8). Trapping of the substrate radical intermediate by O_2 and subsequent reoxidation of the active site ferrous ion leads to the final 13-(*S*)-hydroperoxide product and regeneration of the ferric form of enzyme. EPR studies of the hydroperoxide-activated, ferric form of lipoxygenase indicate signals at $g' = 6$ and 4.3.[9] We have confirmed that anaerobic reduction of this enzyme form with substrate leads to the loss of these EPR signals. Analogous studies, monitored by UV-vis spectroscopy, indicate the loss of a 330 nm species [active, ferric enzyme[10]] following the anaerobic reduction of enzyme by substrate (data not shown).

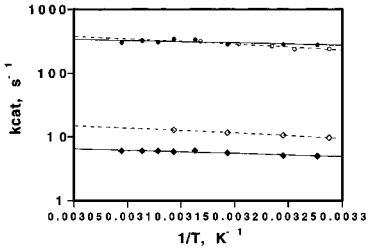

Figure 1. Arrhenius plots of kinetic parameters for lipoxygenase. Maximal rates (k_{cat}) of reaction of linoleic acid (LA) (●) and [$^2H_{31}$]-linoleic acid (D-LA) (◆) under steady state conditions; initial rates were determined spectrophotometrically as published.[6] Maximal rates of reaction for LA (○) and D-LA (◇) under anaerobic, pre-steady state conditions; measurements were made between 31 and 50 °C as described in the legend for Figure 2.

Table 1. Relationship between Activation Parameters and Isotope Effects on Extrapolated Arrhenius Prefactors

enzyme[a]	k_{cat} (s^{-1})[b]	$\Delta H^{\ddagger}$ (kcal/mol)[d] C−H	$\Delta H^{\ddagger}$ (kcal/mol)[d] C−D	A_1/A_2
BSAO	1	12.6	15.2	0.12 (H/T); 0.51 (D/T)
MAO	0.4	12.3	14.5	0.13 (H/T); 0.52 (D/T)
GO	3.7[c]		8.2	1.46 (D/T)
SBL	280	1.2	1.6	27 (H/D)
	>230	3.4	3.1	50 (H/D)

[a] BSAO, bovine serum amine oxidase;[3] MAO, monoamine oxidase B;[4] GO, glucose oxidase (deglycosylated recombinant form).[14] [b] Protium substrate at 25 °C, except for SBL, which is at 32 °C (upper line, steady state), and 31 °C (lower line, stopped flow). [c] For GO, measurements were carried out with deuterated 2′-deoxyglucose. [d] $\Delta H^{\ddagger}$ values are shown for cleavage of protio substrates (left column) and deuterio substrates (right column).

The absorbance decrease at 330 nm during the anaerobic half reaction was thus monitored using rapid mixing stopped flow conditions. The disappearance of absorbance vs. time for D-LA could be fit by a single exponential decay process with a rate constant of 7.8 ± 0.6 s^{-1} at 31 °C (Figure 2B). This rate constant is slightly larger than the value calculated from steady state data[6] but is much smaller than reported in an earlier rapid mixing experiment.[11] Control experiments show that commercial samples of D-LA (containing 1−2% of H-LA) give inflated rate constants under single turnover conditions, due to the preferential reaction of contaminating protio substrate in the millisecond time regime. The reaction of H-LA was more complicated, showing two exponential processes with rate constants of 231 ± 28 s^{-1} and 6.1 ± 0.8 s^{-1} at 31 °C (Figure 2A). The faster rate constant (average of 30 trials) is close to, but slightly reduced from, the steady state value of k_{cat}; additionally, the overall change in absorbance with the protio linoleic acid was approximately half of that seen with deuterio substrate. These features arise because the faster rate process has a half time close to the dead time of the instrument (3 ms). Reduction of the temperature, in order to monitor a greater portion of the H-LA reaction, was not an option, since both pre-steady state (this study) and steady state kinetic studies[6]

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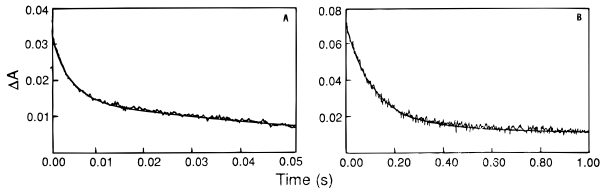

Figure 2. Stopped flow traces of absorbance at 330 nm vs. time for the anaerobic reaction between oxidized (ferric) lipoxygenase and linoleic acid. Data were collected using an Applied Photophysics Limited spectrophotometer. Conditions were 0.1 M borate buffer, pH 9.0, containing 30 μM enzyme and 240 μM substrate, in the presence of 50 mM glucose, 50 units/mL glucose oxidase, and 250 units/mL catalase to maintain anaerobiosis. Reaction of D-LA is shown in B and that of LA in A. D-LA (98% enriched, Cambridge Isotopes) was first reacted with lipoxygenase to remove protium contamination and then repurified by HPLC. The activity of these concentrated samples of lipoxygenase was found to be unchanged during the time required for mixing experiments.

indicate that the C−H bond cleavage step becomes less rate determining below 32 °C. The slower rate constant with the protio substrate corresponds to a relatively minor change in absorbance at 330 nm (amplitude *ca*. one-third of the observable absorbance change) which follows the almost instantaneous formation of the substrate-derived intermediate; this is most likely due to a side reaction of the substrate-derived radical under conditions of strict anaerobiosis. Although an analogous second kinetic process is likely to occur with D-LA, its detection is obscured by the similarity of its rate to that for the C−D bond cleavage step.

The temperature dependence of the pre-steady rate state constants k_H (fast phase) and k_D at 31 °C and above is shown in Figure 1. Although the isotope effects are somewhat reduced and the activation energies (Table 1) somewhat elevated, the data obtained under stopped flow conditions show remarkable similarity to those determined from steady state conditions. This demonstrates that the highly unusual behavior of SBL arises from an innate property of the C−H bond cleavage process.

The behavior of SBL can be understood within the context of the Arrhenius equation. The latter represents a phenomenological description of classical, thermally activated processes, with deviations from Arrhenius behavior arising under conditions of activationless quantum mechanical behavior.[12] Direct experimental observations of curvature in Arrhenius plots due to tunneling are rare; however, a few clear examples at low temperature have been reported.[16−18] Interestingly, all three are for radical reactions, and large isotope effects are reported for the latter two.[17,18] In Figure 3, we have separated Arrhenius plots for a light (L_1) and heavy isotope (L_2) into four regions. Region I corresponds to classical behavior with large enthalpies of activation and extrapolated values for $A_1/A_2 \simeq 1$. The region designated II represents a commonly seen pattern in which isotope effects may be inflated and extrapolated Arrhenius prefactor ratios become less than unity. This behavior, which reflects a greater tunneling and hence curvature in the Arrhenius plot for the lighter isotope, has already been reported in the bovine serum amine oxidase[3] and monoamine oxidase B[4] reactions. Extension of the curves in Figure 3 to a temperature range where significant tunneling of all isotopes of hydrogen takes place leads to nearly activationless processes for both L_1 and L_2 (region IV). In addition to enthalpies of activation close

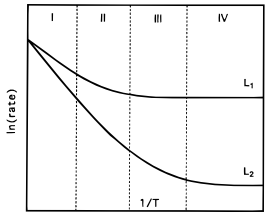

Figure 3. A general diagram describing the rate of a hydrogen transfer reaction as a function of temperature. Two curves (L_1 and L_2) are presented for illustrative purposes; however, these pertain to all three isotopes of H. The regions designated I−IV are described in text.

to zero, this region may lead to extremely large isotope effects and predicts Arrhenius prefactor ratios close to the measured isotope effects themselves (i.e., *all* of the properties seen in the SBL reaction). Several examples of isotope effects on Arrhenius prefactors larger than unity have been reported.[13,19−23] The study of a hydride transfer reaction in solution has shown behavior similar to lipoxygenase, with kinetic isotope effects of 50 that appear as $A_H/A_D \simeq 50$.[13]

The curves in Figure 3 make two predictions: (1) that a system(s) will be found that shows behavior intermediate between regions II and IV and (2) that a trend of decreasing enthalpy of activation will emerge as tunneling becomes more prominent. The properties of intermediate tunneling behavior (region III in Figure 3) are hard to predict, since small changes in rate may lead to values for A_1/A_2 which are either normal or inverse. In fact, recent studies of three glycoforms of glucose oxidase indicate small changes in k_{cat} corresponding to a marked shift in the value of A_1/A_2 from inverse to well above unity.[14] Regarding prediction 2, a correlation between $\Delta H^{\ddagger}$ and A_1/A_2 is now seen among the four enzyme systems for which it has been possible to characterize isotope effects on the C−H bond cleavage step as a function of temperature (Table 1).

What do these results imply regarding the nature of enzyme catalysis? By comparing the observed behavior of lipoxygenase (Figure 1) to that predicted over a wide temperature range (Figure 3), it appears that the active site of lipoxygenase has been optimized to permit extensive barrier penetration by both the light and heavy isotopes of hydrogen. This type of behavior has previously been seen in chemical systems at very low temperatures. Although many models for hydrogen transfer by pure tunneling pathways predict isotope effects considerably in excess of those seen with lipoxygenase, a two-dimensional tunneling model (in which the proton and electron transfers from substrate to the active site $Fe^{III}OH$ center of SBL are strongly coupled) can reproduce the data reported herein.[15] We conclude that the classical view of enzyme catalysis, which has been focused on a reduction in reaction barrier height, must be expanded to include a modification in reaction barrier width. In reality, it appears that the entire potential energy surface at an enzyme active site can be modified with the expectation that the balance between changes in barrier height and width will be a unique property of each system.

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