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Hydrogen Tunneling in Peptidylglycine α-Hydroxylating Monooxygenase

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The assumption of classical, over-the-barrier behavior for hydrogen transfer processes at ambient or higher temperatures in condensed phase has come under increasing scrutiny. To a large extent, this is a consequence of the demonstrated prevalence of tunneling in enzyme-catalyzed C−H activation processes under physiologically relevant conditions.[1,2] As shown for hydride-transfer reactions catalyzed by dehydrogenases, the contributed tunneling can be formalized as moderate.[3,4] In contrast, the hydrogen atom transfer catalyzed by soybean lipoxygenase (SLO) is best described as a full tunneling process that is controlled by a temperature-dependent ligand reorganization (Marcus) term, together with an increased protein gating term for select active-site mutants.[5] For hydrogen-transfer reactions in general, enzymes may optimize catalysis by a reduction in barrier width, given the decreased opportunity for a reduction in barrier height through differential charge stabilizations.

We now describe a study of tunneling in a second enzyme system that catalyzes H• abstraction, peptidylglycine α-hydroxylating monooxygenase (PHM). PHM initiates the oxidative cleavage of C-terminal, glycine-extended peptides by H• abstraction from the α-carbon of glycine.[6] In the PHM reaction, substrate binding and product release contribute significantly to rate limitation under the conditions of steady-state turnover.[7] The criteria for studying tunneling are (1) the kinetic isolation of the hydrogen-transfer step from other partially rate-limiting steps and (2) the ability to examine isotope effects (IEs) on hydrogen transfer as a function of either multiple labeling patterns or temperature.[1] To examine tunneling in the PHM reaction, we turn for the first time to a determination of the temperature dependence of an intrinsic primary IE on the C−H bond cleavage step in an enzymatic reaction.

The determination of intrinsic IEs in the PHM reaction requires precise experimental values for H/D and H/T kinetic IEs. This study uses the smallest peptide substrate that reacts efficiently with PHM, hippuric acid (*N*-benzoylglycine).[8] The observed H/D and H/T primary IEs are shown in Figure 1A. These competitive IEs measurements are for the kinetic parameter k_{cat}/K_M and reflect all steps from substrate binding through the irreversible C−H cleavage step. The reduction in IEs at reduced temperature indicates that substrate activation becomes progressively less rate-limiting, most likely reflecting a larger enthalpy of activation for loss of substrate from enzyme than for the C−H bond cleavage step itself.

The intrinsic deuterium isotope effects are derived using the equation: $\{[^{D}(k_{cat}/K_M) - 1]/[^{T}(k_{cat}/K_M - 1)\} = [(^{D}k - 1)/(^{D}k^{1.442} - 1)]$, in which ^{D}k is the intrinsic deuterium IE and the intrinsic tritium IE is formulated as $^{D}k^{1.442}$.[9] This comes from the Swain−Schaad relationship that assumes classical H transfer. The value of the exponent relating primary H/D and H/T effects is expected to change very little with increasing tunneling.[10] Significantly, in systems demonstrated to proceed with tunneling, the experimental exponential relationship between primary D/T and H/T IEs is seen to be at or very near the semiclassical limit.[3,11−13] Even if the magnitude of the exponent (1.442) were altered for a full tunneling model, it would not be expected to change within the temperature range of this study and, hence, affect any trend in intrinsic isotope effects with temperature.

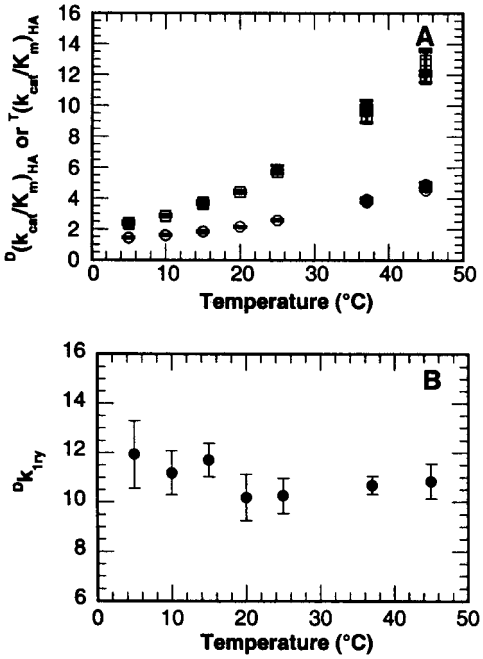

Figure 1. (A) Temperature dependence of $^{D}(k_{cat}/K_m)$ (○) and $^{T}(k_{cat}/K_m)$ (□) with hippuric acid. (B) Temperature dependence of the primary deuterium intrinsic isotope effect for PHM.

The values for the computed intrinsic primary isotope effects are shown in Figure 1B. Two features characterize these data. First, the propagated error envelop increases with decreasing temperature, due to a decrease in the magnitude of the experimentally observed IEs (Figure 1A). Second, there is little apparent change in the intrinsic primary IEs with temperature within experimental error (Figure 1B). The intrinsic α-secondary deuterium IEs were calculated from the experimental secondary tritium IEs, together with the degree of kinetic complexity at each temperature. These show a small trend with temperature, with the average value of the secondary effect being 1.27 ± 0.09.

A fit of the intrinsic primary IEs to the Arrhenius equation for isotope effects: $^{D}k_{1ry} = A_H/A_D \exp\{[E_a(D) - E_a(H)]/RT\}$, yields

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the following parameters: $A_H/A_D = 5.9 \pm 3.2$ and $E_a(D) - E_a(H) = 0.37 \pm 0.33$ kcal/mol.[14] These final values lie well outside of the semiclassical limits ($0.7 < A_H/A_D < 1.4$ and $E_a(D) - E_a(H) =$ 1.2 kcal/mol) and cannot be explained by a tunneling correction.[15−17] The values are, however, characteristic of a reaction in which both the light and heavy isotopes tunnel. A number of examples of this behavior in enzyme reactions now exist in the literature,[5,18] but none has been documented using intrinsic IEs that are free from any possible complication of kinetic complexity.

A second feature of the experimental data is the large magnitude of the intrinsic primary and secondary H/D IEs. The secondary H/D effect is close to the maximum equilibrium IE for conversion of an sp^3 to an sp^2 center, which is anticipated to occur upon abstraction of a hydrogen atom from the α-carbon of glycine, producing a resonance-stabilized radical intermediate. Classically, this implies a very product-like transition state in the PHM-catalyzed H• abstraction.[19] By contrast, the magnitude of the primary H/D IE of 10.5 exceeds the semiclassical maximum value (of 7) and, by virtue of its large value, implies a symmetrical transition state.[20] This behavior, in which the comparative magnitudes of the primary and secondary IEs are incompatible with classical H-transfer has been seen previously in enzyme-catalyzed reactions where tunneling is now well documented.[21]

Overall, the data for PHM provide compelling evidence for nonclassical behavior, introducing another example of an H•-abstracting enzyme that uses tunneling as a primary catalytic strategy. What are the implications of this phenomenon for hydrogen transfer at the PHM active site? The enthalpy of activation for H• abstraction has been proposed to reflect heavy atom motions that control the tunneling coordinate. The kinetic complexity in PHM makes the estimation of $\Delta H^{\ddagger}$ for the C−H cleavage step extremely challenging. We have turned to a method in which it is possible to estimate the rate of an irreversible chemical step using the magnitude of the intrinsic IE (^Dk), together with the experimental IE on k_{cat} ($^Dk_{cat}$): $k_{chem} = [k_{cat}(^Dk - 1)]/(^Dk_{cat} - 1)$.[22]

Choosing two temperatures where the value of $^Dk_{cat}$ is elevated from unity (37 °C, where k_{cat}, $^Dk_{cat}$ and ^Dk are 34.4 ± 0.8 s^{-1}, 1.37 ± 0.19 and 10.4 ± 0.3, respectively, and 15 °C, where k_{cat}, $^Dk_{cat}$ and ^Dk are 4.33 ± 0.12 s^{-1}, 1.25 ± 0.23 and 11.7 ± 0.7, respectively), we estimate the chemical rate to vary from 870 ± 60 s^{-1} at 37 °C to 180 ± 20 s^{-1} at 15 °C. Although there is considerable propagated error in these numbers, there is a clear trend with temperature from which we estimate $\Delta H^{\ddagger}$ of ca. 13 kcal/mol.

In the case of wild-type SLO, the enormous size of the measured IEs, the low experimental enthalpy of activation for C−H cleavage and the near temperature independence of measured IEs imply an active site that has been highly optimized for quantum H-transfer.[5] Starting with the assumption of full nonadiabatic H-transfer in the PHM reaction, we note several features that contrast with SLO; these are the smaller magnitude of the primary kinetic H/D IEs and the significantly larger enthalpy of activation. These features, together with the observation that $A_H/A_D < k_H/k_D$ (5−45 °C), suggest an increased role for protein gating in PHM. We have modeled the data for PHM using the expression for the temperature dependence of the IE derived recently by Kuznetsov and Ulstrup[23] and elaborated in Knapp et al.[5] with the defining parameters being the environmental reorganization energy required to match the energy levels of reactant and product (λ, in kcal/mol) and the frequency of the protein vibration (gating) that controls the H-transfer distance (ω_g in cm^{-1}).

We are able to fit the data for PHM with values for λ and ω_g of 20 kcal/mol and 45 cm^{-1}, respectively (see Supporting Information). For the comparison enzyme, SLO, λ is almost unchanged (19.5 kcal/mol), while ω_g is much larger (400 cm^{-1}).[5] The observed differences between wild-type SLO and PHM most likely reflect very significant differences in active site structures, which are deeply buried in SLO [24] and fully solvent exposed in PHM.[25] The data presented herein indicate a PHM active site that is less optimized for tunneling and more flexible, with greater participation of a gating mode at room temperature. In a recently completed study of mutant forms of SLO,[5] decreases in hydrophobic packing within the enzyme active site have also been found to lead to increases in the role for protein gating on H-transfer. Investigations of this kind can provide unique insight into dynamical motions linked to C−H activation in enzymatic reactions.

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Supporting Information Available: Input parameters and calculated temperature dependence of kinetic isotope effects for PHM and SLO, within an environmentally coupled hydrogen tunneling model (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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