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A Model Reaction Assesses Contribution of H-Tunneling and Coupled Motions to Enzyme Catalysis

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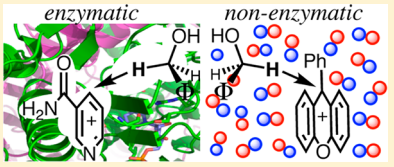
Supporting Information

ABSTRACT: To assess the contribution of physical features to enzyme catalysis, the enzymatic reaction has to be compared to a relevant uncatalyzed reaction. While such comparisons have been conducted for some hydrolytic and radical reactions, it is most challenging for biological hydride transfer and redox reactions in general. Here, the same experimental tools used to study the H-tunneling and coupled motions for enzymatic hydride transfer between two carbons were used in the study of an uncatalyzed model reaction. The enzymatic oxidations of benzyl alcohol and its substituted analogues mediated by alcohol dehydrogenases were compared to the oxidations by 9-phenylxanthylium cation ($PhXn^+$). The $PhXn^+$ serves as an NAD^+ model, while the solvent, acetonitrile, models the protein environment. Experimental comparisons included linear free energy relations with Hammett reaction constant (ρ) of zero versus −2.7; temperature-independent versus temperature-dependent primary KIEs; deflated secondary KIEs with deuteride transfer (i.e., primary-secondary coupled motion) versus no coupling between secondary KIEs and H- or D-transfer; and large versus small secondary KIEs for the enzymatic versus uncatalyzed alcohol oxidation. Some of the differences may come from differences in the order of microscopic steps between the catalyzed versus uncatalyzed reactions. However, several of these comparative experiments indicate that in contrast to the uncatalyzed reaction the transition state of the enzymatic reaction is better reorganized for H-tunneling and its H-donor is better rehybridized prior to the C−H→C transfer. These findings suggest an important role for these physical features in enzyme catalysis.



INTRODUCTION

Catalysis is the ratio of the rate of the catalyzed reaction versus the uncatalyzed one. Much experimental work has been devoted to examining the role of physical mechanistic features in enzymatic H-transfer reactions, including the nature of H-tunneling, the role of protein motions coupled to the reaction, as well as other features presented in refs 1−7 and many others. Yet, the contribution of such features to catalysis was not commonly addressed (even if the wording sometimes suggested otherwise), because a relevant uncatalyzed reaction has not been examined by the same means as the catalyzed one. Several experiments, however, did examine the hydrolytic and C−H bond activation in model systems that are relevant to the enzyme-catalyzed reactions. These include experiments reported by Wolfenden,[8] Kirby,[9] Bruice,[10] Finke,[11] Kreevoy,[12,13] Lee,[14] Frieden,[15] and their co-workers. The first four mentioned here were recently reviewed by Schowen.[5] Some of these studies examined systems that mimic the nicotinamide-dependent enzymes, which are of special relevance to the current study. Two of the mentioned studies by Wolfenden[8] and Finke[11] directly compared their findings to those from the relevant enzymatic reactions (hydrolytic and H-radical metal-catalyzed reactions, respectively).

One enzymatic system where many experiments examined the physical nature of its C−H→C process is the alcohol dehydrogenase (ADH)-catalyzed reaction.[16−26] The reaction involves the reversible oxidation of an alcohol by hydride transfer from the alcohol to the NAD^+ coenzyme. It is a preferred system for physical examination as this chemical conversion would not occur spontaneously (i.e., without a protein catalyst) in physiological solvent. Since the 1970s the mechanistic analysis tools of physical organic chemistry were applied to this system resulting in an in-depth understanding of the enzyme-catalyzed reaction. These experiments include the examination of the linear free energy relationships using substituted benzyl alcohol or aldehyde for the forward and reverse reaction catalyzed by yeast ADH,[16−18] comparison of secondary kinetic isotope effects (secondary KIEs, isotope effect on the hydrogen that is not being transferred) to their equilibrium values (secondary EIE),[19] comparison of secondary KIEs with H- versus D-transfer (denoted primary-secondary coupled motion),[17,20−24] and temperature dependence of primary KIEs with a thermophilic ADH.[25] While at first sight some of the findings from these experiments appeared to contradict each others' conclusions, it was recently suggested that all can be rationalized within the framework of one physical model, referred to below as the Marcus-like model.[26]

The main reason that some of those experimental data appeared contradictory when originally published is that they employed models that are oversimplified. For example, it had been assumed that secondary KIEs indicate the difference

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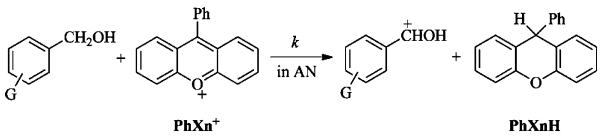
between the hybridization of the labeled carbon (donor or acceptor) at the reactant's ground state versus the hybridization at the transition state (TS). According to this assumption, secondary KIEs should have been between unity (very early TS) and the EIE (very late TS).[27−34] The observation of a normal secondary KIE (>1) for aldehyde reduction, which has an inverse secondary EIE (<1), did not fit that model, leading to proposed H-tunneling effect.[19] Similarly, when secondary D/T KIEs for the enzymatic alcohol oxidation with D-transferred were less than predicted from semiclassical models for secondary H/T KIEs with H-transferred, primary-secondary coupled motion was proposed to correct the simpler model for the deviation from the rule of geometric mean,[4,20,25,35] Additionally, large secondary KIEs for alcohol oxidation indicated late transition state (product-like) in accordance with the equilibrium of that reaction (10:1 [alcohol]:[aldehyde]).[36] The model used in ref 26 suggested that these apparent contradictions could be rationalized in a model that allows for asynchronized hybridization of the donor and acceptor carbon, where the enzyme preorganized the donor (C7 of benzyl alcohol, sp^3 before reaction) for the H-transfer by bringing it closer to sp^2 hybridization at the TS (relative to the classical prediction). According to that model the enzyme reorganized the acceptor (C4 of nicotinamide, sp^2 at the ground state) toward sp^3 prior to H-transfer so that the acceptor's LUMO would be more available to accept the hydride. Furthermore, the thermophilic ADH (*bs*ADH) experiments across a broad temperature range indicate that at its physiological temperatures (30−65 °C) the primary KIEs are temperature-independent.[25] This has been interpreted as requiring an enzymatic TS where the donor and acceptor are well reorganized for H-tunneling, i.e., the enzymes generate short donor−acceptor distance with degenerate donor−acceptor energy levels to promote efficient overlap of the donor's HOMO and the acceptor's LUMO.[37] According to this model the difference in the extent of apparent primary-secondary coupled motions for H- and D-transfers in the enzymatic reaction resulted from the fact that D-tunneling requires a shorter distance relative to H-tunneling.[4] That shorter distance suppresses the secondary KIEs due to steric effects, and this suppression leads to an inflated Swain−Schaad relationship (i.e., ln(secondary H/T KIE with H-transfer)/ln(secondary D/T KIE with D-transfer) leading to the apparent violation of the rule of the geometric mean.[20]

The above enzymatic studies as well as several others[34,38−46] did not fit the semiclassical TS theory leading to the examination of tunneling corrections.[47] Models implying tunneling corrections could reproduce some experimental data better than semiclassical models, but such simple corrections to TS theory cannot reproduce temperature-independent KIEs if the overall rate is temperature-dependent ($E_a > 0$). However, several enzymatic and a few non-enzymatic reactions have exhibited exactly that phenomenon.[48,49] To account for this behavior, many researchers have extended the Marcus theory of electron tunneling[50] to the situation of hydrogen tunneling. The Marcus-like models,[4] also known as full tunneling models,[51] environmentally coupled tunneling,[52] vibrationally enhanced tunneling,[53] configuration-search framework,[5] and other names, suggest that heavy atom motions bring the system to a tunneling ready state where the vibrational energy levels for the hydrogen in the reactant and product are degenerate and tunneling can occur. This type of model gives a rate constant (k) with the functional form

$$k = C(T)\frac{|V|^2}{\hbar}\sqrt{\frac{\pi}{\lambda k_B T}}\, e^{-\frac{(\Delta G^\circ + \lambda)^2}{4k_B T\lambda}} \times \int_0^\infty F(m, DAD)\, e^{-E(DAD)/k_B T}\, dDAD \quad (1)$$

where terms in front of the integral include $C(T)$, which is the fraction of reactive complexes;[35] the electronic coupling between the reactants and products (V); the probability of heavy atom "reorganization" to reach a tunneling ready state,[26] which depends on the reaction driving force (ΔG°), the reorganization energy (λ), and the absolute temperature (T) (k_B is the Boltzmann's constant). In most parts, these terms are insensitive to the mass of the transferred particle, so the terms in the integral bear most of the isotopic sensitivity and correspond to the isotope effects. The integral computes the probability of tunneling to form products once the system reaches the tunneling ready state. The first term inside the integral gives the probability of tunneling as a function of the mass (m) of the transferred particle (H, D, or T in this case) at the tunneling ready state as a function of donor−acceptor distance (DAD). The second term in the integral is a Boltzmann factor giving the probability of reaching a specific DAD. Since the thermal reorganization to reach the tunneling ready state is isotopically insensitive, but tunneling at the tunneling ready state is isotopically sensitive, the model accounts for either temperature-dependent or -independent rates with temperature-dependent or -independent KIEs. It is important to note that these models assume that all the motions of the system are in thermal equilibrium with the environment, and to our knowledge none of these phenomenological models have suggested nonequilibrium or nonstatistical dynamics.[54]

In order to test the hypothesis that protein motions promote H-tunneling and the steric effect (crowdedness) of the active site controls the secondary KIE behavior on the enzymatic H-transfer processes, studies comparing solution uncatalyzed H-transfer reaction are needed.[5,55] In this paper, we present a study of an alcohol oxidation in solution using the same experimental tools and substrate used in studying the enzymatic reaction of the enzyme ADH. We report kinetic studies of the oxidation of benzyl alcohol ($PhCH_2OH$) via hydride transfer to 9-phenylxanthylium ion ($PhXn^+$) in acetonitrile (MeCN) (eq 2). In this reaction, $PhXn^+$ serves as the model



(2)

for NAD^+, and the solvent, acetonitrile, replaced the low dielectric protein environment (based on the closer dielectric constants, 36 for MeCN, in contrast to 78 for water).[56] We determined the Hammett reaction constant for the oxidation of the substituted benzyl alcohols, the secondary OH/OD KIEs on the benzyl alcohol, the secondary H/D KIEs on the same alcohol for both hydride transfer and deuteride transfer, as well as the temperature dependence of primary H/D KIEs for the alcohol oxidation. The results were analyzed following the same analysis that was used for the enzyme-catalyzed reaction.

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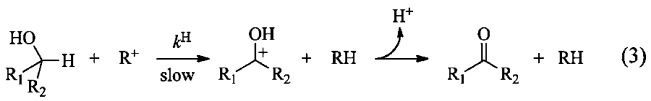
RESULTS

We have recently reported a kinetic and mechanistic study of the oxidation of 2-propanol and 1-phenylethanol via hydride-transfer to carbocationic oxidants (R^+) in various solvent systems to form the corresponding ketone product, the hydride reduction products (RH), and a proton (eq 3, $R_1 = R_2 = CH_3$ in



(3)

2-propanol, and R_1 = Ph, $R_2 = CH_3$ in 1-phenylethanol).[57−60] R^+ used include the $PhXn^+$ (counterion BF_4^-) and the 10-methylacridinium ion (counterion ClO_4^-). Primary H/D KIE, secondary OH/OD KIEs, and secondary β-CH_3/CD_3 KIEs of both alcohols were observed. Both the latter secondary KIEs were normal (KIE > 1), suggesting a hydride-proton sequential transfer mechanism that involves a rate-limiting formation of the α-hydroxy carbocation (C^+-OH) followed by a rapid loss of proton to the basic species (most likely, excess alcohol) in the reaction solution.[57−60]

In this paper, kinetics of the hydride-transfer reactions from the parent and substituted benzyl alcohols (eq 3, R_1 = Ar; R_2 = H) to $PhXn^+$ in MeCN were determined by following the decay of the $PhXn^+$ UV−vis absorption at 373 nm (or 425 nm) with time. The detailed method to determine the pseudo-first-order rate constants of the reaction of alcohols in MeCN was published previously.[58] Kinetics of the reactions of all alcohols with $PhXn^+$ (1.0×10^{-3} M) were determined under pseudo-first-order conditions with alcohol concentrations in large excess (0.02−0.40 M, depending upon the reactivity of individual alcohols), as described before.[60] The effects of [alcohol] on the pseudo-first-order rate constants of the reactions were determined, and all reactions were observed to be first-order in alcohol. The second-order rate constants (k^H) were calculated from dividing pseudo-first-order rate constants by [alcohol].

The UV spectra of the reaction of benzyl alcohol with $PhXn^+$ at different times, i.e., the kinetic scans, are shown in Figure 1.

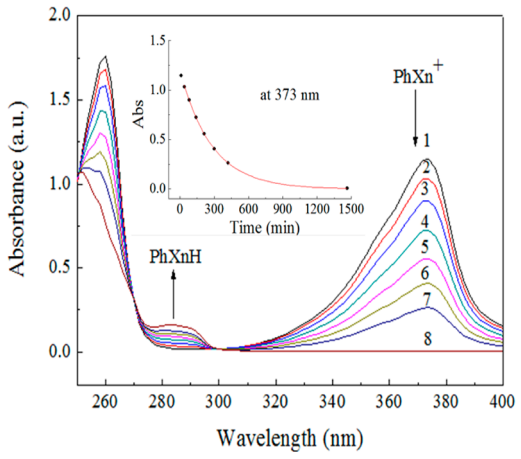


Figure 1. Spectra of the diluted reaction aliquots (by 25 times) taken from the reaction solution of $[PhXn^+]_0$ = 1 mM and $[PhCH_2OH]$ = 60 mM at different times in MeCN at 60 °C. The inset shows the first-order exponential fit of the decay of absorbance at 373 nm with time.

In this figure, the absorption at 373 nm decays due to $PhXn^+$ reduction and the absorption at 285 nm rises due to PhXnH formation.[58,60] Kinetic scans for the reactions of all other substituted alcohols except for the 3-nitro and 4-nitrobenzyl alcohols follow a similar pattern. In the latter two nitro-substituted cases, the absorption of the $PhXn^+$ and that of the large excess alcohols overlap at wavelengths below 400 nm, the absorption decrease at 447 nm due to $PhXn^+$ consumption was then monitored (see Figure 2 for an example).

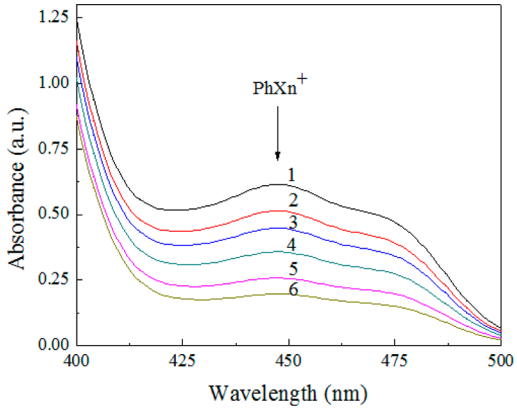


Figure 2. Spectra of the diluted reaction aliquots (by 12.5 times) taken from the reaction solution of $[PhXn^+]_0$ = 1 mM and [p-NO_2-$PhCH_2OH$] = 400 mM at different times in MeCN at 60 °C. Reaction times for spectrum 1 to 6 are 384, 1240, 1907, 2992, 4435, 5800 min, respectively.

Determination of the β-Secondary KIE due to Hydroxyl Deuteration. To further examine the hydride-proton sequential transfer mechanism for the benzyl alcohol oxidation, OH/OD KIE was determined by comparing the pseudo-first-order rate constants of the reactions of the alcohols containing OH or OD under the same conditions. A normal secondary OH/OD KIE value of 1.13 was obtained, consistent with the corresponding β-secondary KIE values found in the reactions of 2-propanol (1.08)[58] and 1-phenylethanol (1.10).[60] This together with the observed primary KIE and α-secondary KIE as well as the linear free energy relation analysis described below for the same system is consistent with the hydride-proton sequential transfer mechanism that involves an α-hydroxy carbocationic TS in the rate-determining hydride transfer step (eq 3).

Linear Free Energy Relation. The Hammett correlation analysis using the second-order rate constants (k^H) was carried out for the oxidation of 12 substituted benzyl alcohols in MeCN at 60 °C (Figure 3). While the Hammett correlation in ADH used σ^+ substituent constants due to the development of the positive charge at the reaction center,[16,17] better linearity was given with σ (R^2 = 0.993) in solution reactions rather than with σ^+ (R^2 = 0.962, see Supplementary Figure S1) even though the observed large negative σ-type Hammett constant (ρ = −2.66) indicates that a significant positive charge develops at the benzylic carbon during the reactions.[61,62] In order to examine the extent of the developing empty 2p orbital for p-π interaction in the reaction systems, we carried out a Yukawa−Tsuno-type correlation analysis.[63] That correlation uses mixed substituent constants [$\sigma + r(\sigma^+ - \sigma)$] in which r reflects the extent of p-π resonance contribution in addition to that defined in that of σ (r = 0 → pure σ-type correlation with both inductive and resonance contribution, whereas r = 1 → pure σ^+-type with extensive additional empty p-π resonance contribution). We found that adding resonance factor by adjusting r does not improve the σ-type correlation at all, suggesting no

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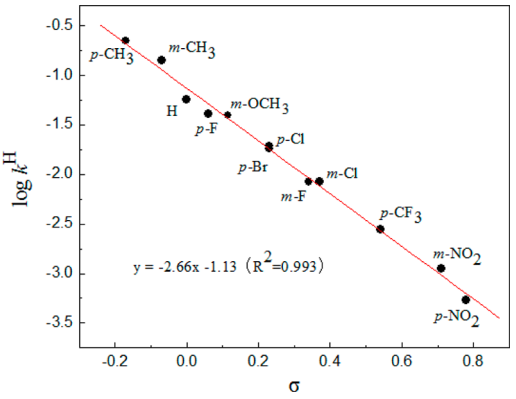

Figure 3. Hammett correlation for the oxidation of substituted benzyl alcohols with $PhXn^+$ in MeCN at 60 °C (k^H in M^{-1} min^{-1}, σ are from refs 64 and 65).

expected amount of additional p-π interaction involved in the TS. The imbalanced hydride transfer TS with the C−H bond largely broken but no matching amount of 2p orbital developed is indeed consistent with our observed secondary KIE results, providing important mechanistic information[66] (see the Discussion and Conclusions sections). Note that the linear free energy relation study has been carried out for the alcohols oxidation by chromate salts in the past,[67,68] but to the best of our knowledge, this is the first Hammett analysis study of alcohol oxidation by an organic carbocation via a pure hydride transfer representing C−H→C transfer of direct relevance to the enzyme catalysis.

Determination/Derivation of Primary H/D KIE and α-Secondary H/D KIE. Since there are two hydrogens (H_i and H_j) at the benzylic carbon, we use the notation of k_i^j to represent the rate constant for H_i that is transferring (primary-H) and H_j that is staying (secondary-H). The notation of k_{ij} is used to represent the observed overall rate constant for the reaction in which the transfers of H_i and H_j both contribute. Therefore, for the reaction of $PhCH(i)H(j)OH$, $k_{ij} = k_i^j + k_j^i$, and for the reaction of $PhCH_iH_iOH$, $k_{ii} = 2k_i^i$. For KIEs, we define $KIE_{mn/op} = k_m^n/k_o^p$. Thus,

$$\text{primary KIE}_{HH/DD} = k_H^H/k_D^D \quad (4)$$

$$\text{primary KIE}_{HH/DH} = k_H^H/k_D^H \quad (5)$$

$$\text{secondary KIE}_{HH/HD} = k_H^H/k_H^D \quad (6)$$

$$\text{secondary KIE}_{DH/DD} = k_D^H/k_D^D \quad (7)$$

Note that the KIEs defined in eqs 6 and 7 are the secondary H/D KIEs for H- and D-transfers, respectively.

To determine the $KIE_{HH/DD}$, we compare the second-order rate constants k_{HH} (= $2k_H^H$) and k_{DD} (= $2k_D^D$) determined with $PhCH_2OH$ and $PhCD_2OH$. This KIE combines primary and secondary effects, and its conversion to real primary KIE (e.g., k_H^H/k_D^H) is presented below. Similarly, using $PhCH_2OH$ and PhCHDOH, we can determine a mixed primary/secondary $KIE_{HH/(HD+DH)} = 2k_H^H/(k_H^D + k_D^H)$. Here "mixed" refers to the combination of k_H^H versus k_H^D (secondary H/D KIE) and k_H^H versus k_D^H (primary H/D KIE). Since $k_H^D \gg k_D^H$ (by a factor close to the primary KIE), this measurement yields value that is between the secondary KIE and the primary KIE (Table 1).

The expected normal secondary KIEs for the $sp^3 \rightarrow sp^2$ conversion (e.g., k_H^H/k_H^D or k_D^H/k_D^D) cannot be determined directly but can be derived from the two observed KIEs ($KIE_{HH/DD}$ and $KIE_{HH/(HD+DH)}$) together with the measurable concentration ratio of the H-transfer product and D-transfer product for the reaction of PhCHDOH, i.e., [PhCHO]/[PhCDO] or [PhXnH]/[PhXnD]. This latter ratio is equal to k_H^D/k_D^H. Then, substituting the observed k_H^D/k_D^H into observed $k_H^H/k_D^D = 3.74$ and $2k_H^H/(k_H^D + k_D^H) = 1.67$ will give rise to k_H^H/k_H^D and k_D^H/k_D^D, the secondary KIEs on both H- and D-transfer processes.

We determined the [PhXnH]/[PhXnD] ratio using ^1H NMR technique. We compared the integrated areas (A) of the 9-H and 2,7-HH peaks for the mixture products of PhXnH and PhXnD from the reaction of PhCHDOH at 60 °C (Supplementary Figure S3, also see Supplemnetary Table S2 for the NMR data of PhXnH product isolated from the reaction of $PhCH_2OH$ for comparison). According to the relationship following our mechanism (eq 3) $2A_{9\text{-H}}/A_{2,7\text{-HH}}$ = [PhXnH]/([PhXnH]+[PhXnD]) = $k_H^D/(k_H^D + k_D^H)$. Since the PhXnH and PhXnD are produced simultaneously from the same reaction mixture, and since the $PhXn^+$ is achiral, the [PhXnH]/[PhXnD] should be independent of the reaction time.[69] The $A_{2,7\text{-HH}}/A_{9\text{-H}}$ values were determined for the reaction aliquots at reaction times at 30 min, 90 min, 120 min, and 24 h using the same integration method as used in the above control experiment with $PhCH_2OH$ (Supplementary Figure S3). As expected, the $A_{2,7\text{-HH}}/A_{9\text{-H}}$ ratios (with an average of 2.67 ± 0.03) are constant within the experimental error. The determination was repeated twice and gave rise to k_H^D/k_D^H of 2.99 ± 0.03. The secondary $KIE_{HH/HD}$ (for H-transfer) and secondary $KIE_{DH/DD}$ (for D-transfer) were then calculated (see the Supporting Information for the calculations of both KIEs and standard deviations). In addition, since $KIE_{HH/DD} = (k_H^H/k_D^H)(k_D^H/k_D^D)$, i.e., primary $KIE_{HH/DH}$ × secondary $KIE_{DH/DD}$, the primary $KIE_{HH/DH}$ can be calculated using $KIE_{HH/DD}/KIE_{DH/DD}$. This latter primary KIE excludes the secondary KIE factor included in the observed primary $KIE_{HH/DD}$. Both observed and calculated KIE values and the corresponding standard deviations are presented in Table 1.

Temperature Dependence of Primary KIEs. The $KIE_{HH/DD}$ values were determined at temperatures ranging from 22 to 67 °C. The primary $KIE_{HH/DH}$ values at different temperatures are therefore calculated from dividing $KIE_{HH/DD}$ values by secondary $KIE_{DH/DD}$ (1.12) derived above (Table 1,

Table 1. KIEs of the Hydride-Transfer Reaction from Benzyl Alcohol to $PhXn^{+a,b}$

$KIE_{HH/DD}$[c]	$KIE_{HH/(HD+DH)}$[c]	$KIE_{HD/DH}$[d]	secondary $KIE_{HH/HD}$[e]	secondary $KIE_{DH/DD}$[e]	primary $KIE_{HH/DH}$[f]
3.74 ± 0.14	1.67 ± 0.05	2.99 ± 0.03	1.11 ± 0.06	1.12 ± 0.09	3.3 ± 0.3

[a]In MeCN at 60 °C. [b]$KIE_{mn/op} = k_m^n/k_o^p$; m,o are primary H(D), and o,p are secondary H(D). See text for detailed definitions. [c]From spectroscopic kinetic determination. [d]From ^1H NMR determination. [e]Secondary KIEs and standard deviations (SDs) are calculated using observed KIEs on HH/DD, HH/(HD+DH), and HD/DH (see text and Supporting Information). [f]Equal to $KIE_{HH/DD}/KIE_{DH/DD}$; the SD is calculated using $SD_{HH/DH} = KIE_{HH/DH}((SD_{HH/DD}/KIE_{HH/DD})^2 + (SD_{DH/DD}/KIE_{DH/DD})^2)^{1/2}$.

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secondary KIE does not change significantly with temperature, as they are too small to significantly affect the temperature dependence of primary KIEs). The change in this primary KIE across the temperature range, the Arrhenius preexponential ratio (A_H/A_D), and the isotope effect on activation energy ($\Delta E_{a(D-H)} = E_{aD} - E_{aH}$) are given in Table 2.

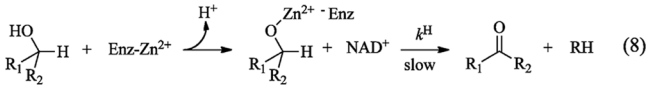
Table 2. Primary KIEs of the Hydride-Transfer Reactions from Benzyl Alcohol to PhXn$^+$ in MeCN as a Function of Temperature and the Isotope Effect on Their Activation Parameters

temp (°C)	$KIE_{HH/DD}$ [a]	primary $KIE_{HH/DH}$ [b]	activation parameters
67	3.83 ± 0.25	3.4 ± 0.3	uncatalyzed reaction:[c]
60	3.74 ± 0.14	3.3 ± 0.3	A_H/A_D = 0.8 ± 0.3
54	4.02 ± 0.16	3.6 ± 0.3	$\Delta E_{a(D-H)}$ = 4.2 ± 1.1 kJ/mol
45	3.99 ± 0.22	3.6 ± 0.3	
36	4.31 ± 0.08	3.9 ± 0.3	enzymatic reaction:[d]
29	4.38 ± 0.27	3.9 ± 0.4	A_H/A_D = 2.2 ± 1.1
22	4.83 ± 0.21	4.3 ± 0.4	$\Delta E_{a(D-H)}$ = 2.1 ± 2.5 kJ/mol

[a]This work. [b]See footnote f in Table 1. [c]This work, derived from the temperature dependence of k_H^H and k_D^H, corresponding to the column of primary $KIE_{HH/DH}$ in this table (see Table S4 in Supporting Information); [d]From ref 25 for the *bs*ADH at 30−65 °C.

DISCUSSION

To use model reactions to provide insight into the mechanism of the ADH reactions studied, it is important to compare their reaction mechanisms (eq 3) with that found in Zn containing ADHs, where the enzyme-bound Zn^{2+} first catalyzes the deprotonation of the alcohol to form an intermediate Zn-alcoholate followed by hydride transfer to the NAD^+ cofactor (eq 8).[70−72] Since in both cases the intermediate complex is of



higher energy, the hydride transfer step for the model system is endothermic (step 1 in eq 3), while the enzymatic one is exothermic[26] (step 2 in eq 8). Consequently, data were collected in a fashion that enabled separation of effects that result from the differences in the mechanisms from those effects that result from differences in the nature of the C−H→C transfer. To clarify, it is the nature of the hydride transfer in enzyme that is compared, rather than the simplified kinetic terms that are composed of many microscopic rates and equilibrium constants such as V/K and k_{cat} (the second- and first-order rate constants of the Michaels equation).[73]

The role of phenomena such as H-tunneling, protein dynamics, and the stabilization of TSs in enzyme-catalyzed reactions has been studied extensively. However their contribution to the ratio of catalyzed to uncatalyzed rates (i.e., catalysis) remains elusive due to a paucity of studies of uncatalyzed reactions. To assess the contributions of various physical features to enzyme catalysis, we applied tools previously used for enzymatic C−H→C transfers to an uncatalyzed model reaction. Because of the difference in the sequence of the proton and hydride abstraction steps in the uncatalyzed reaction and the enzymatic alcohol oxidation (eq 3 vs eq 8), the experimental findings are discussed both with respect to the different order of transfer step and with respect to differences in the nature of the hydride transfer.

Experimental Finding Associated with Mechanistic Order−Linear Free Energy Relation. The hydride transfer in the uncatalyzed reaction (eq 3, step 1) is endothermic whereas the hydride transfer in the enzymatic reaction (eq 8, step 2) is exothermic. The observed Hammett reaction constant (ρ) of −2.66 for the model reaction implies a late TS on the hydride transfer coordinate. This is very different from what was found in the linear free energy relation study of the yeast ADH reaction that gave a reaction constant (ρ^+) close to zero, suggesting a very early, i.e., alcohol-like TS.[16,17] The hydride transfer process for the latter enzymatic reaction is exothermic due to the high reactivity of the zinc alkoxide reactant, which is in accordance with an early TS as indicated by the corresponding Hammett correlation results.[26] Moreover, since the positive charge developed at the benzylic carbon during the hydride transfer is more readily delocalized to the Zn-alkoxide, the substituent effect is further reduced.

Experimental Finding Associated with the Hydride Transfer Step: KIEs. *Temperature Dependence of Primary KIEs.* As apparent from Table 2, the size of the primary KIEs and their temperature dependence for the uncatalyzed reaction can be understood using semiclassical TS theory, i.e., no tunneling correction (KIE 3.4−4.3; A_H/A_D = 0.8 and $\Delta E_{a(D-H)}$ = 4.2 kJ/mol). These findings can be explained by differences of zero point energies and the Bigeleisen model,[74] with no need to invoke H-tunneling. The thermophilic *bs*ADH at similar temperature range (including 60 °C, its physiological growth temperature) showed A_H/A_D >1 (2.2) and temperature-independent KIEs ($\Delta E_{a(D-H)}$ = 2.1 kJ/mol).[25] The comparison of the uncatalyzed with catalyzed reactions in the framework of a semiclassical TS theory involving a simple tunneling correction would suggest no H-tunneling for the uncatalyzed and extensive H-tunneling for the catalyzed reactions.[51]

The above analysis would have suggested that tunneling makes a significant contribution to enzyme catalysis. However, in the framework of this model such a conclusion is not justified since the enzymatic reaction has a significant enthalpy of activation, which would not predict temperature-independent KIEs.[4,24,25,37,75,76] On the other hand, Marcus-like models could explain both temperature-dependent and temperature-independent KIEs for reactions with significant enthalpy of activation.[4,37,77,78] Such analysis suggests a very different reorganization in these two reactions. The enzymatic one has a short DAD that is well oriented for H-tunneling, while the non-enzymatic H-transfer occurs with a longer DAD and the two reactants are less well organized for H-tunneling at the tunneling ready state. This analysis indicates that relative to the uncatalyzed reaction, the enzyme better orients the H-donor and acceptor and "compresses" them to a short DAD, to enable H-tunneling.

Secondary KIEs as Indicators for the Rehybridization of the H-Donor. In the ADH-catalyzed reaction secondary H/T KIEs with H-transfer were compared to secondary D/T KIEs with D-transfer and analyzed in the context of Swain−Schaad exponent (secondary SSE)[27] and the rule of the geometric mean.[51,79,80] That analysis implied both primary-secondary coupled motion and H-tunneling and explained the inflation of secondary H/T KIEs as being due to more extensive H-tunneling in the case of secondary H/T KIEs relative to D-tunneling in the case of secondary D/T KIEs, which leads to inflated secondary SSE.[20] It was later realized that the inflation

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of secondary SSEs actually resulted from deflated secondary D/T KIEs in those experiments.[81] It was then suggested that the basis of the explanation of the deflated secondary D/T KIEs and inflated secondary SSEs is that D-tunneling only occurs from shorter distances than H-tunneling. Since the ADHs evolved to catalyze H-transfer rather than D-transfer, the shorter DAD for D-transfer leads to steric effects that limit the rehybridization at the TS and result in deflated secondary KIEs when D is in the primary position. This analysis was in good agreement with recent calculations based on the Marcus-like models of secondary KIEs of yeast ADH.[26]

The secondary KIEs observed in this work for the $PhXn^+$ oxidation of benzyl alcohol were the same for H and D transfers (Table 1). This finding is in accordance with the rule of the geometric mean and with non-semiclassical H-tunneling theory. Nevertheless, in order to compare this finding to the equivalent enzymatic observations, which are not in accordance with semiclassical approaches, different analysis is required. As discussed above, and within the framework of the Marcus-like models, the current observation indicates a long DAD for both H and D transfers, leading to no sterically hindered rehybridization for the reaction with D-transfer and thus no primary isotope effects on the secondary KIEs. This observation supports the hypothesis that enzyme motions evolved to bring the reactants to much better orientation for H-tunneling, more restrictive dynamics at the tunneling ready state, and shorter DAD than those found in the MeCN solvent reaction.

A comparison of the relations between the secondary KIEs and the secondary EIE in solution versus enzyme systems is also informative. In the enzyme ADH, the oxidation is exothermic, suggesting an early TS (by the Hammond postulate). The linear free energy relation analysis discussed above is in agreement with this postulate (similar electronic state at ground state and TS for alcohol oxidation);[17] however, the secondary KIEs were as large as the secondary EIE, which semiclassically would suggest a late TS. This apparent contradiction was recently reconciled in the context of the Marcus-like models,[26] indicating that the rehybridizations of the donor and acceptor carbons are not synchronized, and H-tunneling occurs from a state where both carbons were closer to their rehybridization at the product state (relative to the classical prediction). In the model reaction, on the other hand, the C−H→C hydride transfer is endothermic (late TS by Hammond postulate) but the secondary KIE (1.11, Table 1) was only about half of the secondary EIE (1.26[82,83]), which by semiclassical TS theory would indicate an intermediate TS. This seems to suggest that the rehybridization of the H-donor lags behind the H- transfer, consistent with the imbalanced TS structure predicted by the linear free energy relation analysis in which observed large negative Hammett constant contradicts with the σ-type of correlation (see Results section). This comparison suggests that the enzyme leads to more changes in the donor and acceptor hybridization as part of the reorganization prior to H-tunneling, while in the solvent the H-transfer occurs from a TS whose formation precedes the rehybridization of the H-donor.

The observed relationships among non-semiclassical secondary KIE, secondary EIE, and Hammond postulate in the benzyl alcohol oxidation is also found in other non-enzymatic systems. We recently reported[58,60] a β-secondary KIEs close to unity for the endothermic reactions of 2-propanol (β-d_6 KIE = 1.06) and 1-phenylethanol (β-d_3 KIE = 1.03) with $PhXn^+$ in MeCN. The relevant EIE for the 2-propanol reaction is estimated to be 1.52.[84,85] Thus by semiclassical theory, the much smaller secondary KIE close to unity would suggest an early TS consistent with the current findings with benzyl alcohol oxidation. These findings suggest that the nonclassical hydride transfer behavior might be a common feature for this class of hydride transfer reactions.

CONCLUSIONS

The same mechanistic experimental tools used to study the H-tunneling and coupled motions for ADH-catalyzed H-transfer in the benzyl alcohol oxidation were used to study an uncatalyzed model reaction. These comparative experiments include linear free energy relation studies, measurements of primary KIEs and their temperature dependence, and measurements of secondary KIEs with H-transfer and with D-transfer (i.e., secondary KIE with H or D in the primary position). The linear free energy relation studies are consistent with the proposed difference in the sequence of mechanistic steps of the enzymatic versus uncatalyzed hydride transfer under study and find the H-transfer step to be exothermic for the enzyme and endothermic for the uncatalyzed reactions. The KIE experiments, on the other hand, seem to reveal differences in the C−H→C transfer step *per se*.

The comparison of temperature dependence of primary KIEs for the *bs*ADH-catalyzed reaction to the uncatalyzed model reaction over similar temperatures indicates little or no temperature dependence for the enzyme reaction and a significant dependence for the uncatalyzed reaction (Table 2). In the framework of semiclassical TS theory, the first would suggest extensive tunneling and the second little or no tunneling, i.e., indicating an extensive tunneling contribution to catalysis. Within the framework of Marcus-like models, the enzyme reorganizes the reaction coordinate so that H-tunneling occurs at short average DADs with narrow distribution (i.e., resulting from a high "gating" frequency or well organized dynamics), while in the solvent the average DAD is too long for efficient tunneling. The broader distribution allows for population of shorter DADs resulting in smaller primary KIEs at higher temperatures, i.e., temperature-dependent KIEs.

One of the most broadly used experimental test for non-semiclassical features in ADHs has been inflated secondary SSE, and deviation from the rule of the geometric mean. For the uncatalyzed reaction with the same substrate, we found no evidence for such inflated secondary SSE, i.e., no violation of the rule of the geometric mean or other predictions from semiclassical models. Within the framework of semiclassical TS theory with tunneling correction, this difference suggests a unique contribution of primary-secondary coupled motion and tunneling to enzyme catalysis. Within the framework of Marcus-like models, however, it suggests that enzyme motions bring the donor and acceptor to a much closer approach, and a much higher "gating" frequency than the uncatalyzed reaction, showcasing the role of these features in catalyzing H-tunneling.

Another clue that enzyme catalysis involves careful reorganization of the reaction for H-tunneling is the mismatch of the extent of the hydride transfer at the TS (early or late TS) with the relative magnitudes of secondary KIEs (in between unity and their secondary EIE values). The catalyzed alcohol oxidation exhibits a small Hammett constant (reactant-like TS) but EIE like secondary KIEs (product-like hybridization, by semiclassical TS theory), whereas the uncatalyzed reaction reported here shows the opposite. The TS of the ADH-catalyzed

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reaction is early; however, both donor and acceptor carbons are already closer to products in rehybridization. In the uncatalyzed reaction, however, the rehybridization lags behind the formation of the TS, emphasizing again the role of the enzyme in arranging the reaction coordinate as major feature in catalysis.

In summary, the comparative studies of benzyl alcohol oxidation by enzymes with an uncatalyzed model reaction reveal important physical features of catalysis. To examine how general these findings are, more model systems have to be tested using the same experimental tools used for their enzymatic counterparts. Additionally, better understanding of the differences of enzyme-catalyzed reactions and their uncatalyzed counterparts at the molecular level would require more effort in calculating and simulating the model reaction with methods like QM/MM/MD simulations. Better understanding of the contribution of such physical features to catalysis could have significant implications to our understanding of early enzyme evolution, catalysis in non-enzymatic systems, and the design of biomimetic catalysts.

EXPERIMENTAL SECTION

General. All chemicals were purchased and used as are, unless indicated. Acetonitrile (MeCN) for kinetic determination was redistilled twice (first time over P_2O_5) in the N_2 atmosphere to ensure dryness. The 9-phenylxanthylium tetrafluoroborate ($PhXn^+BF_4^-$) was synthesized by reacting 9-phenyl-9-xanthenol with tetrafluoroboric acid according to the published procedure.[86] The racemic benzyl alcohol-α,α-h,d (PhCHDOH) and benzyl alcohol-α,α-d,d ($PhCD_2OH$) were synthesized from the reactions of benzaldehyde and benzoyl chloride with $LiAlD_4$ in dry tetrahydrofuran, respectively.[87] In order to exclude the effects of different impurities from various sources on the accurate determination of the KIEs, the unlabeled benzyl alcohol ($PhCH_2OH$), though commercially available, was also made by reduction of the benzaldehyde with $LiAlH_4$. The benzyl alcohol-O-d ($PhCH_2OD$) was prepared by deuterium exchange of the OH group in benzyl alcohol with DCl in D_2O in the N_2 atmosphere. The deuterium content in the deuterated benzyl alcohols was found to be higher than 99.5% per C−D bond according to the ^1H NMR analysis.

General Kinetic Determination Procedure.[58] A 40 μL portion of 0.1 M stock solution of $PhXn^+$ in MeCN was added to 4 mL of MeCN solution containing large excess of alcohol in a sealed 10 mL reaction vial that was placed in a water bath set for a desired temperature. Aliquots of about 0.25 mL were periodically withdrawn from the reaction into sample vials precooled in ice. The samples were immediately placed in a freezer (~ −20 °C). For each experiment, 7−8 reaction aliquots were collected within 1−3 half-lives of the reaction. The 80 μL aliquots of the reactions were then diluted into 1.92 mL of MeCN in a cuvette for spectral analysis. The corresponding UV spectra at different reaction times, i.e., the kinetic scans, were then obtained. Absorbance (A) decreasing with time (t) at 373 or 447 nm due to the $PhXn^+$ consumption was recorded as a function of time (e.g., Figures 1 and 2). The obtained $A-t$ data were fit to the integrated first-order rate equation, $-\ln(A) = kt$ + constant, and the slope of the linear plot of $-\ln(A)$ versus t was taken as the pseudo-first-order rate constant of the reaction. The linear plots usually had regression coefficients (R^2) greater than 0.997 (most often above 0.999). The effect of alcohol's concentration on pseudo-first-order rate constants was determined and the reactions were found to follow a second-order rate law. Second-order rate constants (k^H) were derived for Hammett analysis (Supplementary Table S1).

The KIEs were obtained by taking the ratios of the second-order rate constants observed for the reactions involving normal and deuterium-labeled benzyl alcohols ($PhCH_2OH$ vs $PhCL_2OH$, L = H or D), respectively. The rate constants were measured in duplicates to tetraplicates and primary KIEs calculated (Supplementary Table S4). For determination of the small secondary KIEs, parallel determination of the rate constants of the reactions of normal and deuterated alcohols were conducted in hexaplicates (Supplementary Table S3).

Determination of the ^1H NMR of the PhXnH/PhXnD Mixture for the Reaction of PhCHDOH. The reaction was carried out using the same procedures for kinetic determinations. The 0.12 mmol of PhCHDOH and 0.04 mmol of $PhXn^+$ in 4 mL MeCN was added to a 10 mL reaction vial placed in a water bath of 60 °C. The reaction was stopped at a desired reaction time by adding water. The reaction product was then extracted with methylene chloride. The extracts were washed with water and dried over anhydrous $MgSO_4$ before rotatory evaporation. The PhXnH/PhXnD mixture was separated through column chromatography and analyzed by ^1H NMR.

ASSOCIATED CONTENT

Supporting Information

^1H and ^{13}C NMR spectra of PhXnH(D) from the reaction of $PhCH_2OH$ and PhCHDOH and the raw kinetic data for the Hammett correlation analysis and to derive the KIEs and Arrhenius parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(84) Estimated from the reported β-secondaro EIE (1.28)[82] for the conversion from 2-propanol to acetone and that from acetone to the protonated acetone (1.19).[85]

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