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Imbalanced tunneling ready states in alcohol dehydrogenase model reactions: rehybridization lags behind H-tunneling[†]

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The secondary kinetic isotope effects for the hydride transfer reactions from aliphatic alcohols to two carbocations (NAD⁺ models) in acetonitrile were determined. The results suggest that the hydride transfer takes place by tunneling and that the rehybridizations of both donor and acceptor carbons lag behind the H-tunneling. This is quite contrary to the observations in alcohol dehydrogenases where the importance of enzyme motions in catalysis is manifested.

The structure of the transition state (TS) for chemical reactions is central to the understanding of the reaction mechanisms as well as to the design of enzyme inhibitors and drugs. One important tool in investigating the TS structure is the secondary kinetic isotope effect (2° KIE) that results from the isotopic substitution on a bond that is not breaking during the reaction.¹⁻³ 2° KIE originates from the differing bonding/hybridization in the reactant state and the TS, which are manifested in vibrational frequency changes, which will differ for isotopes.¹⁻³ Relative to the complete hybridization changes (reactant to product) associated with a secondary equilibrium isotope effect (EIE), this change is only partial, suggesting that 2° KIEs fall in between unity (without hybridization change) and the 2° EIE. Thus, traditionally, a 2° KIE close to unity suggests that the TS resembles the reactant (i.e. early TS) and a 2° KIE close to the 2° EIE implies that the TS resembles the product (i.e. late TS).^{1,3–5} While such correlation between 2° KIE and EIE has been well applied to the mechanistic understanding of the reactions involving heavy atom bond changes,^{3,4,6-8} its application in the study of the light H particle transfer reactions has been challenging.^{9,10} Since the early 1980's, 2° deuterium or tritium (H/D or H/T) KIEs for many H-transfer reactions, both in solution and in enzymes, have been observed to be larger than the value predicted by the classical TS, and even outside of the range between unity and 2° EIEs.9-16 This has been explained in terms of the 1°/2° H coupled motions and 1° H-tunneling, in which part of the 2° H bond out of plane bending vibrational modes are converted to a translational mode, leading to an inflated 2° KIE as a result of partial loss of bending vibrations.17,18 Recent explanation for this abnormal 2° KIE behavior on hydride transfers in the

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dihydrofolate reductase (DHFR) and alcohol dehydrogenases (ADHs) was, however, based on the variational TS theory¹⁹ and the Marcus-like H-tunneling model.²⁰ Results suggest that H-tunneling accounts for the inflation of the 2° KIEs, and it involves an imbalanced TS or "tunneling ready states (TRS's)" (Fig. 1) with asynchronous H donor and acceptor rehybridization.^{19,20} At the TRS's, the reactants are already rehybridized closer to the products, preparing favorable orbital conditions for the hydride to transfer/tunnel, i.e., rehybridization precedes the hydrogen tunneling.²⁰ It has been suggested from these findings that enzymes reorganize the reaction coordinate for efficient H-tunneling by advancing the necessary geometry change as well as shortening the donor acceptor distance through physical motions/vibrations. This is consistent with the recent proposals from many other studies that enzyme motions are coupled to the chemistry step thus contributing to catalysis.²¹⁻²³

The above proposal about an enzyme's special function in organizing the reaction coordinate for efficient H-tunneling can be tested by comparing the findings in enzymes with those in their model reactions. One support is from our recent report of an observed imbalanced TRS for the hydride transfer reaction from benzyl alcohol to 9-phenylxanthylium ion (PhXn⁺) in acetonitrile (MeCN) (eqn (1), R1 = R = Ph, R = H).²⁴ This was treated as a model reaction of enzymes that mediate hydride transfers between two carbons and is best described as a model for alcohol dehydrogenases (ADH's) that mediate hydride transfer from alcohol to NAD⁺.²¹ It was found, by comparison of the α -2° KIE on benzyl alcohol (PhCL₂OH, L = H or D, from sp³ to sp²) with the corresponding EIE, that rehybridization occurs after the hydride tunneling at the TRS. This is opposite to the order found in enzymes, strongly supporting the above important proposal



Fig. 1 Energetic comparison of species involved in a H-transfer reaction from donor (D) to acceptor (A). In the TRS, H transfers in terms of its wave properties through a donor–acceptor distance longer than that in the classical transition state. The non-filled area in TRS indicates H wave properties delocalized between D and A.

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regarding the role of protein motions in catalysis.²⁴ In order to strengthen the conclusion, it is necessary to expand the study to other H-transfer systems in solution, especially those in which the 2° KIEs on both the H-donor and H-acceptor can be determined so that the rehybridization of both can be examined. For this purpose, use of the xanthylium ion (Xn⁺) as a hydride acceptor is necessary since the 2° KIE on its 9-C–L bond (sp² to sp³) can also be determined. In this paper, we study the hydride transfer reactions from three aliphatic alcohols to the Xn⁺ as well as to the PhXn⁺ in MeCN (eqn (1)). The β -D₆ 2° KIEs at the 2CH₃/ $2CD_3$ position of 2-propanol and the α -D 2° KIEs at the 9-H/D position of Xn^+ were determined. Our observations include: (1) the 2° KIEs on alcohols were close to unity and those on Xn⁺ were close to or even larger than unity, and (2) the 2° KIEs on hydride transfer were close to or slightly larger than that on deuteride transfer. The results elude explanation by the traditional TS theory. These can only be explained by assuming hydride tunneling accompanied by asynchronous rehybridizations of both hydride donors and acceptors and asynchronous development of charge and rehybridization in forming the TRS.



The kinetic procedure and the mechanistic study of the reaction of alcohols with $PhXn^+BF_4^-$ in MeCN were previously reported.²⁴⁻²⁷ Here, kinetics of the hydride-transfer reactions from alcohols to $Xn^+ClO_4^-$ in MeCN were similarly determined by following the decay of the Xn^+ UV-Vis absorption (Fig. S1, ESI†). The effects of [alcohol] on the pseudo-first-order kinetics of the reactions were also determined, showing first-order dependence on [alcohol]. The KIE on the OH/OD position of the 2-propanol was determined. The observed normal 2° OH/OD KIE (1.02) together with the 1° α -H/D KIEs (see Table 1) on the alcohol suggests that the reactions use the same hydride–proton sequential transfer mechanism, which involves a rate-limiting formation of an α -hydroxy carbocation intermediate (C⁺–OH) (eqn (1)) followed by its rapid deprotonation to form the C==O compound.

The β -2° CH₃/CD₃ KIEs on hydride transfer and deuteride transfer processes were determined by comparing the second-order rate constants of the reactions of 2-propanol and 2-propanol- β -d₆, and those of the reactions of 2-propanol- α -d₁ and 2-propanol- α -d₁- β -d₆, respectively. The α -2° H/D KIEs at the 9-H/D position of Xn⁺ on the hydride transfer (with normal 2-propanol) and deuteride transfer (with 2-propanol-d₁), were determined by comparing the rate constants of the reaction of the normal Xn⁺ (Xn(9-H)⁺) with that of the 9-deuterated Xn⁺ (Xn(9-D)⁺). The KIE measurements were repeated at least eight times on different days and the averaged KIEs are reported. These together with the 1° KIEs calculated are listed in Table 1.

While the $2^{\circ} \beta$ -D₆ KIEs on 2-propanol in its hydride transfer reaction to both Xn⁺ and PhXn⁺ are normal, the $2^{\circ} \alpha$ -D KIEs on Xn⁺ in the reactions of all alcohols are either normal or inverse but close to unity (Table 1). In order to estimate the position of the TS on the reaction coordinate, comparison of the KIEs to the corresponding EIE is needed. The β -D₆ 2° EIEs on 2-propanol can be estimated from the

Table 1 1° and 2° KIEs for the hydride transfer reactions from alcohols to Xn^+ and Ph Xn^{+a}

Alcohol	CL ₃ CY(C	OH)CL ₃		CH ₃ CY(OH)-	с–(CH ₂) ₅ - СХОН
Cation	$Xn(L)^+$		PhXn ⁺	$\operatorname{Xn}(L)^+$	$Xn(L)^+$
1° KIE ^b	2.75 (0.10)		3.26 (0.14)	2.45 (0.18)	2.36 (0.11)
2° KIE ^c	β - H_6/D_6	α - H/D	β - H_6/D_6	α - H/D	α - H/D
Y = H	1.05	0.99	1.05	1.03	1.00
	(0.02)	(0.01)	(0.04)	(0.02)	(0.02)
Y = D	1.04	Ò.98	1.00	Ò.99	Ò.98
	(0.02)	(0.03)	(0.03)	(0.02)	(0.01)
2° EIE ^d	1.52	0.89	1.52	0.89	Ò.89
^{<i>a</i>} In MeCN at 60 °C numbers in parentheses are standard deviations					

 b L = H, Y = H or D. c β-H₆/D₆ (on 2-propanol), α-H/D (on Xn⁺). d Estimated, see text.

reported β -2° EIE (1.28)²⁸ for the conversion from 2-propanol to acetone and that from acetone to protonated acetone $(1.19)^{29}$ The calculated EIE of 1.52 (= 1.28×1.19) for the conversion from CH-OH to C⁺-OH is consistent with an $sp^3 \rightarrow sp^2$ process. The α -D 2° EIE at the 4-H/D position of the central pyranium ring of Xn⁺ can be assumed to be comparable to the reported α -D 2° EIE at the 4-H/D position of the NAD⁺ pyridinium ring (0.89, for $sp^2 \rightarrow sp^3$).¹³ While the error in the estimated α -D 2° EIE on Xn⁺ is expected to be small, that in the estimated β -D₆ 2° EIE on 2-propanol may be relatively large as the value is derived from the two EIEs from two research groups using different calculation methods. Nevertheless, a comparison of the 2° KIEs with the corresponding range from unity to the corresponding EIEs (1.05 vs. 1.00 to 1.52 and 0.98 \sim 1.02 vs. 0.89 to 1.00) indicates that both KIEs are very close to unity and thus it is safe to describe them to be far from the corresponding EIEs. This suggests early reactant-like TS's. This, however, is inconsistent with the endothermic rate-determining hydride transfer process that forms a highly reactive C^+ -OH intermediate (eqn (1)), which suggests a late TS according to Hammond's Postulate.²⁴ The late TS proposal is also consistent with our reported large negative Hammett reaction constant ($\rho = -2.66$) for the hydride transfer reactions from substituted benzyl alcohols to PhXn⁺ in MeCN.²⁴ The contradiction indicates that at the reaction TS, the α-C-H bond in alcohol has cleaved to a large extent but the rehybridization of both carbons lags behind, implicating that the reaction involves a TS with imbalanced development in charge and rehybridization.

The above mentioned observed inflated 2° KIEs on Xn⁺ (far from the EIE of 0.89 or even exceeds unity for an *endothermic* reaction) (Table 1), on the other hand, provide strong support for a non-classical H-tunneling mechanism. Similar results were observed in enzyme reactions and explained by suggesting a H-tunneling mechanism.^{9–16} For example, in the yeast ADH that mediates the *exothermic* hydride transfer from the reactive zinc alkoxide to NAD⁺, it was observed that a 2° H/D KIE on the 4-H/D position of the NAD⁺ was larger than unity (1.08), which is outside the normal range (0.89 (EIE) to unity) for a sp² to sp³ conversion.¹³ Moreover, 2° KIE on the α -H/D position in benzaldehyde for its reduction by NADH mediated by the yeast ADH (1.05) was also found to be inflated.²⁰ These results were explained by H-tunneling effects, $1^{\circ}/2^{\circ}$ H coupled

motions and asynchronous rehybridizations of the donor and acceptor (*i.e.*, rehybridization precedes H-tunneling).^{9–17,19,20}

While the 2° KIEs on Xn⁺ are inflated, those on the alcohol side are, however, deflated; *i.e.*, $2^{\circ} \beta$ -D₆ KIEs on 2-propanol are close to unity (1.00-1.05), which should be close to the EIE of 1.52 for an *endothermic* reaction with a late TS (Table 1). These elude explanation by the traditional model of H-tunneling and $1^{\circ}/2^{\circ}$ H coupled motions that can only explain the inflated 2° KIEs for H-transfer reactions (see Introduction), but they can be interpreted by the Marcus-like H-tunneling model.^{21,30–36} Within the latter model, the magnitude of the 2° KIE depends upon the extent of the reorganization of the 2° H/D toward the formation of the TRS.³⁶ Thus, both the deflated (close to unity) 2° KIEs on alcohol and also the aforementioned inflated (also close to unity) 2° KIEs on Xn⁺ can be explained in terms of the small degree of reorganization of 2° H/D (equivalent to a small degree of rehybridization) incurred in forming the TRS. In this context, our observed slightly larger 2° KIE on H⁻ transfer than on D⁻ transfer (Table 1) can be interpreted as due to the relatively longer donor-acceptor tunneling distance in H⁻ transfer so that more room is allowed for reorganization resulting in the larger 2° KIE. Note that this explanation on the basis of the difference in H- and D-tunneling distances has been successfully used to explain the observed deflated 2° D/T KIE on alcohol for D⁻ transfer as compared to the 2° H/T KIE for H⁻ transfer in ADH mediated alcohol oxidations.^{21,36} For example, the $\log(k^{\rm H}/k^{\rm T})_{\rm H}/\log(k^{\rm D}/k^{\rm T})_{\rm D} = 3.26$ (superscript (2°), subscript (1°)) predicted for the classical mechanism was observed to be as large as 10.³⁷ The current explanation is that, in enzymes that are evolved for the transfer of the most abundant H isotope, the D-transfer requires shorter tunneling distances, thus inducing crowded/ deformed active sites and decreasing the reorganization of the 2° isotope and the magnitude of the 2° KIE.^{21,36} It should be emphasized that while this 1° isotope effect on 2° KIEs was significant in the ADH systems, it was observed to be very small or even insignificant (within experimental errors) in our solution reactions (Table 1). This may be explained in terms of the loose TRS structures in a less restricted solution medium so that the effect of tunneling distance of H and D on 2° KIEs is greatly minimized.24

To summarize, kinetics of the hydride transfer reactions from three aliphatic alcohols to two NAD⁺ models (PhXn⁺ and Xn⁺) were determined. 2° KIEs on both hydride donors and acceptors for both hydride transfer and deuteride transfer were determined. Both of them are close to unity and significantly far from the corresponding EIEs. 2° KIE on 9-H/D of Xn⁺ in some systems are even larger than unity outside of the normal range from EIE to unity. Moreover, 2° KIEs are slightly larger in hydride transfer than in deuteride transfer in some systems. These findings definitely suggest a non-classical hydride tunneling mechanism involving an imbalanced tunneling ready state where rehybridization of both the hydride donor and the acceptor lags behind the H-tunneling. This is in sharp contrast to the H-tunneling mechanism found in the corresponding enzymatic reactions (e.g., the ADH reactions) in which rehybridization precedes H-tunneling. The latter observations in enzymes have been used to suggest the role of enzyme motions in organizing favorable reaction geometries for H-tunnelling, which should contribute to the

enzymatic rate enhancement. The findings in this work lend strong support to this important proposition.

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