Imbalanced Transition States from **α**-H/D and **β**-Type N-CH/D Secondary Kinetic Isotope Effects on the NADH/NAD$^+$ Analogues in Their Hydride Tunneling Reactions in Solution

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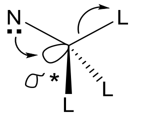
ABSTRACT: The α-H/D (if available) and β-type N-CH$_3$/CD$_3$ 2° KIEs on 10-methylacridine (MAH), 9,10-dimethylacridine (DMAH), 1,3-dimethyl-2-phenylbenzimidzole (DMPBIH) and oxidized forms (D)MA$^+$ in their hydride transfer reactions with several hydride acceptors/donors in acetonitrile were determined. The corresponding equilibrium isotope effects (EIEs) were computed. Hammett correlations of several closely related hydride transfer reactions were constructed using the literature data. The α-2° KIEs on both MAH and MA$^+$ are inflated relative to the semiclassical prediction on the basis of the KIE/EIE comparison and Hammond's postulate. This together with previously published unusual 1° and 2° KIE behaviors strongly suggest a H-tunneling mechanism. By comparing with the EIEs, the α-2° KIEs were used to analyze the rehybridization of the reaction C-center and the N-CH$_3$/CD$_3$ 2° KIEs to calculate the charge distribution on the structure containing N during H-tunneling. The rehybridization appears to lag behind the charge development in the donor moiety. The charge distribution at the tunneling ready TS is in agreement with the Hammett correlations; the donor is product-like and acceptor is reactant-like, indicative of partial negative charge borne by the "in-flight" nucleus being "hydridic" in character. Results were compared with the α-2° KIEs on NADH/NAD$^+$ and the Hammett correlations in closely related enzymes. The comparison implicates that the H-tunneling probability would be enhanced by these enzymes.

Secondary (2°) hydrogen (H) kinetic isotope effect (KIE) is important to the understanding of the structure of the transition state (TS).[1,2] In organic reactions, most reported 2° KIEs are at the α- or β-H/D positions of the C reaction centers. By comparison with the equilibrium isotope effect (EIE), 2° KIE has been used to estimate the position of the TS on the reaction coordinate. It is expected to fall in the range from unity to EIE. When it is closer to unity, the TS is reactant-like; when closer to EIE, the TS is product-like.[1,3-8] This empirical prediction, however, often fails for H-transfer reactions, especially in enzymes. One of the earliest such observation was in 1984 by Cleland and coworkers who found a normal α-2° KIE of 1.23 at the 4-H/D position of the NAD$^+$ in its oxidation of formate ion via hydride transfer catalyzed by a formate dehydrogenase.[9] The KIE value for a sp^2 to sp^3 conversion, which is supposed to be inverse, is outside of the EIE-unity range. This was then explained by a H-tunneling mechanism in which the 2° H/D vibrations are coupled with the 1° H-tunneling.[10,11] The explanation was confirmed by Huskey and Schowen as well as Saunders by theoretical calculations.[12,13] Subsequently, study of the 2° KIEs on a variety of enzyme catalyzed H-transfer reactions was carried out to prove or disapprove the H-tunneling effect and 1°/2° H coupled motions.[14-22] In the meantime, other H-tunneling models were also attempted to explain the inflated 2° KIEs.[19,21-25] Moreover, a comparison of 2° KIEs on some enzymes *vs.* their model reactions in solution has showed that the 2° KIEs in solution were less inflated or even not inflated, making enzymologists conclude that the H-tunneling is enhanced by enzymes.[26,27] On the other hand, other workers also provided evidence that H-transfer in both solution and enzymes take place by H-tunneling effects, but no apparent enhancement of the H-tunneling was shown in enzymes.[28,29]

We have recently made a comparison of the 2° KIEs on the oxidation of alcohols with NAD$^+$ mediated by alcohol dehydrogenases with those on the model reactions.[23,30] The model reactions are the hydride transfers from alcohols to xanthylium ion (Xn$^+$BF$_4^-$) and its 9-phenyl substituted analogue (PhXn$^+$BF$_4^-$) that mimic the NAD$^+$. Several nonclassical behaviors of the KIEs were found, including; inconsistency with Hammond's postulate, and inflated 9-α-2° KIEs on Xn$^+$ but deflated 2° KIEs on alcohols (α-2° KIE on benzyl alcohol or β-2° KIE on isopropyl alcohol). (Here, inflated and deflated could mean being relative to the predicted classical values and not necessarily outside of the unity-EIE range.) These behaviors, together with the observed other nonclassical behaviors of 1° and 2° KIEs in these reactions suggest H-tunneling effect.[23,31] Together with the Hammett correlations, we found that the rehybridization in alcohol lags behind the charge development in the tunneling TS, which is opposite to the order in enzymes.[23] This implicates that enzymes reorganize the reaction coordinate for efficient H-tunneling by advancing the necessary geometry change through protein motions. While this is a start to systematically compare the 2° KIEs on between NAD$^+$ and model reactions, there are still many unanswered questions. (1) The NAD$^+$ models above are (Ph)Xn$^+$ that use O to replace N in NAD$^+$. What are the 2° KIEs on the close NAD$^+$ analogues containing N? (2) The α-2° KIE on the NADH donor was found to be inflated in the dihydrofolate reductase of *Escherichia coli* (*ec*DHFR).[32] What is the behavior of 2° KIEs on the NADH models? (3) While the imbalance of rehybridizations in between donor and acceptor has been observed both with enzymes and models involving H-tunneling, what is the difference in charge development on the donor *vs.* acceptor? (4) Lastly, the β-type CH/D KIE on the other reaction center N has never been studied on either NADH/NAD$^+$ or analogues. What mechanistic information can this KIE bring us?

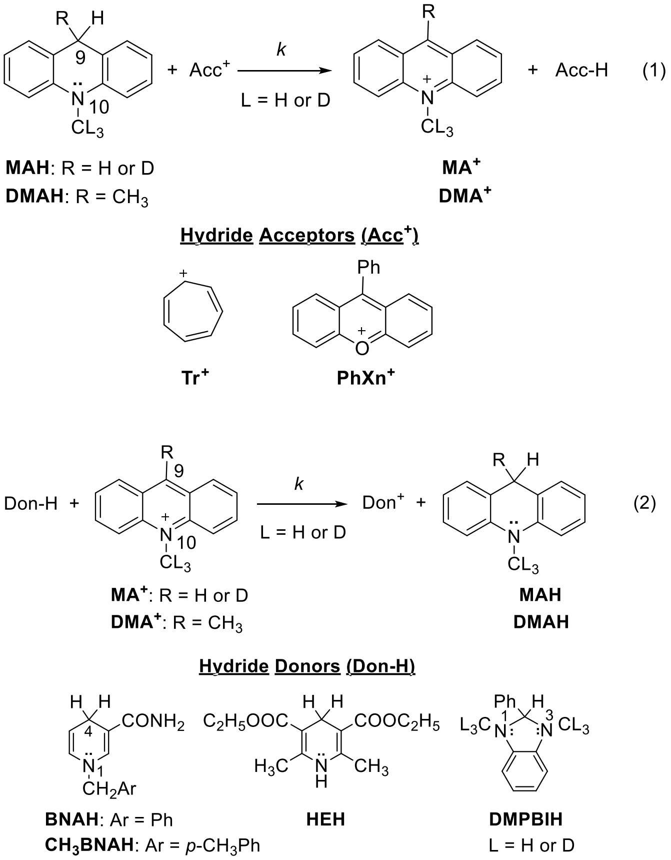
The last question is particularly interesting. To our best knowledge, the β-N-CH/D KIE has not been reported on any reactions by far, although the N-CH/CD isotope effects for the protonation of CL_3-NR_2 (L = H or D) amine bases have been long known.[33,34] The latter are EIEs not KIEs though. It was found that the CD_3 base is stronger than the CH_3 base, leading to EIE < 1. Current explanation uses the concept of negative hyperconjugative effect between the lone pair electrons in sp^3 hybridized N and the empty σ* orbital of the β-C-L bond.[35,36] This effect weakens the C-L bond (via S_N2-like mechanism, Scheme 1). When CL_3-NR_2 is protonated becoming CL_3-N^+HR_2, this effect disappears strengthening the C-L bond thus resulting in an inverse EIE. Therefore, a comparison of the N-CH/D KIE with EIE could give information about the charge development at the N during the reaction. Note that in these protonation processes the hybridization of N is not expected to change (sp^3 to sp^3).



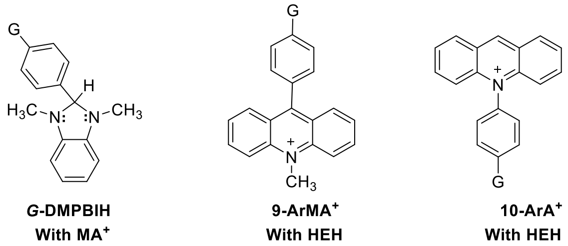
Scheme 1. Negative hyperconjugation between the lone pair electrons on N and the empty σ* orbital of the β-C-L bond weakens the said bond (L = H or D)

In this paper, we studied a series of NADH/NAD^+ model reactions in attempt to answer the questions above. We determined the 2° N-CH/D KIEs (β-type) on the N center of the NADH/ NAD^+ analogues in their hydride transfer reactions in acetonitrile. We also determined the 2° H/D KIEs at the α-C center of the compounds, if available. The NADH/NAD^+ analogues for the KIE study include 10-methylacridine (MAH) and its oxidized form 10-methylacridinium ion (MA^+) (counter ion: BF_4^-, below same), their 9-methyl substituted structures, 9,10-dimethylacridine (DMAH), 9,10-dimethylacridinium ion (DMA^+), as well as 1,3-dimethyl-2-phenylbenzimidazole (DMPBIH) (eqn. (1) in Scheme 2). The (D)MAH, (D)MA^+ and DMPBIH have N-CH_3/CD_3 groups but only the MAH/MA^+ contain the α-H/D group. In the (D)MAH reactions (eqn. (1A)), the hydride acceptors include tropylium ion (Tr^+) and 9-phenylxanthylium ion ($PhXn^+$). In the (D)MA^+ reactions (eqn. (1B)), the hydride donors include 1-(substituted)benzyl-1,4-dihydronicotinamide (*G*BNAH) (*G* = H and CH_3), Hantzsch ester (HEH), and DMPBIH. Note that these hydride donors and acceptors (except Tr^+) are also close NADH/NAD^+ analogues. The reactions are all exergonic (see Results section) and kinetic studies of all of them have been reported.[31,37-40] We also computed the relevant EIEs to compare with the KIEs. Furthermore, by using the literature data,[40] we constructed the Hammett correlations for both the donor and acceptor of the hydride transfer reactions, from 1,3-dimethyl-2-(para-substituted phenyl)benzimidazole (*G*-DMPBIH) to MA^+, from HEH to 9-(para-substituted phenyl)-MA^+ (9-$ArMA^+$) and to 10-(para-substituted phenyl)-MA^+ (10-$ArMA^+$), in acetonitrile (Scheme 3).

The sign (normal or inverse) and magnitude of the 2° KIEs, the comparison between the KIEs and EIEs, the relationship between KIE/EIE comparison and predictions from Hammond's postulate, the synchronicity between the charge development at N (or on the N-ring) and the rehybridization at the α-C, as well as the charge development in between the donor and acceptor in the TS will be discussed, both semiclassically and within a H-tunneling mechanism. The N-CH_3/CD_3 KIE/EIE comparison will be used to calculate the charges on the donor and acceptor moieties. The resulting charge distribution at the hydride tunneling TS for the reaction between DMPBIH and MA^+ will be compared with those gained from the Hammett correlations (Scheme 3). These results will be compared with the α-2° KIEs and Hammett correlations for the hydride transfer reactions involving NADH/NAD^+ in the closely related enzymes. The possible role of these enzymes in H-tunneling and catalysis will be discussed.



Scheme 2. The hydride transfer reactions studied in this work; The α-2° H/D and β-type 2° N-CH/D KIEs on (D)MAH, (D)MA^+ and DMPBIH were determined.



Scheme 3. Hammett analysis of the reaction systems to derive the electronic structure of the TS for the hydride transfer reaction from DMPBIH to MA^+ in acetonitrile. This is used to compare with the same information gained from the N,N-2CH_3/2CD_3 2° KIE on DMPBIH and N-CH_3/CD_3 2° KIE on MA^+ for their reaction in this work.

Results

The 2° KIEs at the 10-N-CH_3/CD_3 of (D)MAH and at the 1,3-N,N-2CH_3/2CD_3 KIE of DMPBIH as well as the α-2° KIEs at the 9-CH/CD of MAH for their reactions with different hydride acceptors in acetonitrile are listed in Table 1. Note that the α-2° KIE on MAH for its reaction with Tr^+ has been reported by us but has not been used to answer the questions raised in this paper.[31] The 2° KIEs at the 10-N-CH_3/CD_3 of (D)MA^+ and the α-2° KIEs at the

9-CH/CD of MA^{+} for their reactions with various hydride donors in acetonitrile are listed in Table 2.

The relevant EIEs calculated for (D)MAH and DMPBIH to release a hydride ion and for $(D)MA^{+}$ to accept a hydride ion in acetonitrile medium are also listed in Tables 1 and 2, respectively, for comparison with the corresponding KIEs. The hybridization states computed in these structures are listed in Table 3. The gas-phase EIEs were also calculated (see *SI*). The values between the two media only differ by less than about 3‰ per CH_3 group. The level of theory used is B3LYP/6-31+G*. We also used the MP2/cc-pVTZ level of theory for some of the calculations and found that the resultant EIEs are consistent. The comparison can be found in Table S15 in *SI*.

Table 4 includes the free energy changes (ΔG°) of some reactions in acetonitrile calculated from the hydride affinity data of the relevant carbocations in literature.[40] The data show that our reactions are exergonic reactions.

Lastly, Figure 1 shows the Hammett correlation constructed from the literature data for the hydride transfer reactions from 1,3-dimethyl-2-(para-substituted(G)phenyl)benzimidazole (*G*-DMPBIH) to MA^{+} in acetonitrile.[40] The Hammett plots for the other two systems in the same solvent, HEH with 10-arylsubstituted acridinium ions ($10\text{-}ArA^{+}$), HEH with 9-aryl substituted MA^{+} ($9\text{-}ArMA^{+}$), can be seen in *SI* (Figures S1, S2).[40] The Hammett constants (ρ's) for all of these reactions are listed in Table 5.

Table 1. The β-2° KIEs at the N-CH_3/CD_3 of (D)MAH and DMPBIH and the α-2° KIEs at the 9-CH/CD of MAH for their reactions with different hydride acceptors as well as the corresponding EIEs [a]

H-acceptors	2° Isotope Effects on H-donors	
	at 10-N-CH_3/CD_3	at 9-CH/D
	2° KIEs on MAH	
Tr^{+} [c]	0.92 (0.04) [b]	1.09 (0.04) [c]
	2° EIEs on MAH for releasing a hydride ion [d]	
	0.883 [e]	1.109 [e]
	2° KIEs on DMAH	
Tr^{+} [c]	0.93 (0.03) [b]	N/A
$PhXn^{+}$	0.92 (0.02) [f]	N/A
	2° EIEs on DMAH for releasing a hydride ion [d]	
	0.905	N/A
	at 1,3-N,N-$2CH_3/2CD_3$	at 2-CH/D
	2° KIEs on DMPBIH	
MA^{+}	0.89 (0.01) [f]	N/A
DMA^{+}	0.88 (0.01) [b]	N/A
	2° EIEs on DMPBIH for releasing a hydride ion [f]	
	0.814	N/A

[a] In acetonitrile, numbers in parentheses are standard deviations; [b] at 35 °C; [c] from ref[31]; [d] at 25 °C, computed with acetonitrile as medium, using the polarizable continuum model (PCM); the level of theory used is B3LYP/6-31+G*; [e] also calculated at higher MP2/cc-pVTZ level, the results are consistent (see Table S15 in *SI*); [f] at 24 °C.

Table 2. The 2° KIEs at the 10-N-CH_3/CD_3 of $(D)MA^{+}$ and the α-2° KIEs at the 9-CH/CD of MA^{+} for their reactions with different hydride donors as well as the corresponding EIEs [a]

H-donors	2° Isotope Effect on H-acceptors	
	at 10-N-CH_3/CD_3	at 9-CH/D [b]
	2° KIEs on MA^{+} [c]	
BNAH	1.05 (0.01)	1.02 (0.01) [d]
CH_3BNAH	1.02 (0.01)	1.01 (0.01)
HEH	1.01 (0.01) [e]	1.02 (0.01) [e]
DMPBIH	1.01 (0.02)	1.00 (0.01)
	2° EIEs on MA^{+} for accepting a hydride ion [f]	
	1.133 [g]	0.902 [g]
	2° KIEs on DMA^{+}	
HEH	1.02 (0.01) [h]	N/A
DMPBIH	1.01 (0.01) [i]	N/A
	2° EIEs on DMA^{+} for accepting a hydride ion [f]	
	1.105	N/A

[a] In acetonitrile, numbers in parentheses are standard deviations; [b] the D content in $MA(9\text{-}D)^{+}$ is 95% by NMR, the 9-CH/D 2° KIE values are not changed after correction; [c] at 24 °C; [d] 0.99 (0.02) was reported in ref[31] (see Table S9 in SI); [e] at 20 °C; [f,g] see footnotes d and e in Table 1; [h] at 30 °C; [i] at 35 °C.

Table 3. Hybridization states of C-9 and N-10 in (D)MAH and $(D)MA^{+}$ [a]

	MAH	MA^{+}
at C_9	$sp^{3.00}$	$sp^{2.02}$
at N_{10}	$sp^{2.31}$	$sp^{2.11}$
	DAMH	DMA^{+}
at C_9	$Sp^{2.92}$	$sp^{2.05}$
at N_{10}	$sp^{2.07}$	$sp^{2.03}$
	DMPBIH	$DMPBI^{+}$
at C_2	$Sp^{2.99}$	$sp^{2.00}$
at N_1,N_3	$sp^{2.62}$	$sp^{2.11}$

[a] At 25 °C, same as footnotes d and e in Table 1.

Table 4. The free energy changes of some hydride transfer reactions in acetonitrile at 25 °C [a]

Donor	MAH	MAH	BNAH	HEH	DMPBIH
Acceptor	Tr^{+}	$PhXn^{+}$	MA^{+}	MA^{+}	MA^{+}
ΔG° (kcal/mol)	- 6.5	- 15.4	- 16.9	- 11.8	- 23.5

[a] Calculated from the hydride affinity data for the corresponding cations from literature[40]

Table 5. The Hammett constant (ρ) for some hydride transfer reactions in acetonitrile [a]

Donor	*G*-DMPBIH [b]	HEH [c]	HEH [c]
Acceptor	MA^{+}	$9\text{-}ArMA^{+}$	$10\text{-}ArA^{+}$
ρ	- 2.78	1.28	1.47

[a] Constructed from the data in literature, at 25 °C;[40] [b] see Figure 1; [c] see *SI* for Hammett plots

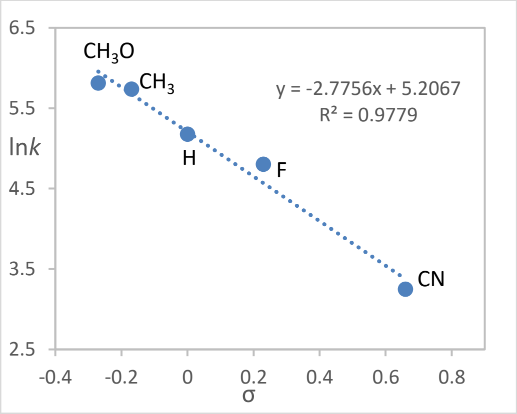


Figure 1. Hammett correlation for the hydride transfer reactions from 1,3-dimethyl-2-(para-substituted(G)phenyl)benzimidazole (*G*-DMPBIH) to MA^{+} in acetonitrile (Constructed from the data in literature[40])

Discussion

The normal 9-α-H/D EIE value (1.109) for the change from MAH to MA^{+} is consistent with an sp^3 to sp^2 conversion (Table 1 for eqn. (1A), also see the revered EIE in Table 2 for the sp^2 to sp^3

process in eqn. (1B)). The inverse 2° N-CH$_3$/CD$_3$ EIEs (0.91 - 0.81) for the conversion from (D)MAH/DMPBIH to (D)MA$^+$/DMPBI$^+$ are consistent with the "negative conjugative effect" explanation, which uses the concept that the CD$_3$-N base is stronger than the CH$_3$-N base (Table 1 and Scheme 1, also see the reversed EIE in Table 2 for the eqn. (1B) process for comparison).[35,36] The 10-N-CH$_3$/CD$_3$ EIE on the conversion from MAH to MA$^+$ (0.883) is more inverse than that from DMAH to DMA$^+$ (0.905) (Table 1). This can be interpreted in terms of more electron density localized on N in MAH available for the negative hyperconjugative effect (due to less planar sp$^{2.31}$ hybridization) than in DMAH (with sp$^{2.07}$ hybridization) (Table 3). *I.e.*, the higher electron density is on N, the more inverse the 2° EIE will be. As far as the DMPBIH is concerned, it has two CH$_3$/CD$_3$ groups and the hybridization change on N is the largest (from sp$^{2.62}$ to sp$^{2.11}$) (Table 3), so the N,N-2CH$_3$/2CD$_3$ EIE has the largest inverse value (0.814). Note that the 9-α-H/D 2° EIE value (1.109) on MAH/MA$^+$ agrees well with that of the 4-H/D EIE (1.12) on NADH/NAD$^+$ that was experimentally determined.[11] In order to further ensure that our computed EIEs are safe to be used to compare with the observed KIEs for mechanistic information derivation, we computed the EIE for a model system closer to the NADH/NAD$^+$ conversion, which is the conversion from 1-methyl-1,4-dihydronicotinamide (MNAH) to its oxidized form MAN$^+$. The α-2° EIE at the 4-H/D position was found to be 1.111, which is close to the experimental value of EIE on NADH/NAD$^+$ (see Table S15 in *SI*).

Semiclassically, the α-2° CH/D KIE is due to the hybridization change at C-9 (change between sp^2 and sp^3), and the β-type 2° N-CH/D KIE is resulted from the change in electron density (charge) at N (N-10 in (D)MA and N,N-1,3 in DMPBI). This is based on the assumption that there is no steric effect contribution to the KIEs from other structures in the TS that can affect the CH/D vibrations. We have confirmed this for the reactions of MAH/MA$^+$; the small-sized α-H/D is not readily accessible to other structures in the TS. Moreover, since the N-CH$_3$/CD$_3$ group is remote from the reaction center, steric effect on its C-H/D vibrations from other structures would be expected to be minimal, too. Therefore, a comparison of the two types of the KIEs with their EIEs will be expected to provide information about the rehybridization and charge development in donor or acceptor, respectively, as well as the balance/imbalance of rehybridization and charge development in between donor and acceptor during the reaction. It should be noted that, if H-tunneling is involved, its known possible contribution to the α-2° KIEs must be considered in the KIE/EIE comparison for a correct analysis of the position of the TS.[25] Below the KIE/EIE comparisons will be discussed both semiclassically and within a H-tunneling mechanism.

Preliminary semiclassical analysis of the 2° KIEs

The N-CH$_3$/CD$_3$ 2° KIEs for the reactions of (D)MAH and DMPBIH with all hydride acceptors are inverse and similar (0.92 – 0.93 for (D)MAH, 0.88 – 0.89 for DMPBIH) (Table 1). The KIEs at N-10 of (D)MAH for its reactions with Tr$^+$ and PhXn$^+$ are close to each other but the ΔG°'s of the two reactions differ by about 9 kcal/mol (Table 4). This indicates that the 2° KIE is not quite sensitive to the acceptor structures used. The inverse values are consistent with the "negative hyperconjugation" explanation (Scheme 1, again the CD$_3$-N base is stronger.). All of the KIEs are closer to EIE as compared to unity. This suggests that the (D)MA/DMPBI moiety of the TS's resemble the (D)MA$^+$/DMPBI$^+$ product in electronic structure, *i.e.* they carry closer to 1+ charge relative to a neutral structure. Moreover, the 9-α-2° KIE on MAH for its reaction with Tr$^+$ (1.09) is also closer to the corresponding EIE (1.109). This normal value is consistent with a conversion from sp^3 to sp^2 hybridization. Semiclassically, this suggests that the changes in hybridization at C-9 and in electron density at N-10 of MAH are largely synchronous in the reaction coordinate, *i.e.*, both are consistent with a late TS. The late TS is, however, inconsistent with the exergonic reactions that have a suggested TS resembling the reactant structure according to Hammond's postulate.

When (D)MA$^+$ are used as hydride acceptors, the 2° KIEs at N-CH$_3$/CD$_3$ become normal (1.01 – 1.05, Table 2). This is because the resulting stronger CD$_3$-N base makes (D)MA$^+$ harder to accept a lone pair of electrons. The 2° KIEs are similar (except for BNAH (1.05) and CH$_3$BNAH (1.02) (without apparent reason)), suggesting that the 2° KIEs would not be quite sensitive to the donor structures, either (*c.f.* ΔG°'s in Table 4). They are close to unity, suggesting a very early TS in charge development (the positive (+) charge on (D)MA moiety in the TS is closer to 1+ charge.). This appears consistent with the exergonic nature of the reactions, *i.e.*, consistent with Hammond's postulate that suggests an early TS. However, the normal α-2° KIEs at the 9-H/D position of MA$^+$, although close to unity (1.00 – 1.02), are inconsistent with the hybridization change from sp^2 to sp^3. The KIEs are out of the range from unity to EIE (0.904), which cannot be used to evaluate the position of the TS in the reaction coordinate. It should be noted that the α-9-H/D 2° KIEs on Xn$^+$ in its reactions with different alcohols were also observed to be close to or slightly larger-than-unity.[30] For example, for the reactions with isopropyl alcohol, 3-methyl-2-butnaol and cyclohexanol in acetonitrile, the α-2° KIEs on Xn$^+$ are 0.99, 1.03, 1.00, respectively. These 2° KIEs are out of the range from unity to EIE as well.[30]

The interesting finding here is that in terms of both the hybridizations and charge development on the (D)MA and DMPBI moieties, the TS's tend to be product-like in the reactions of MAH and DMPBIH (eqn. (1A)) but reactant-like in the reactions of (D)MA$^+$ (eqn. (1B)). Semiclassically, this suggests that the rehybridization and charge development in between the donors and acceptors in this type of hydride transfer reactions, for example between DMPBIH and MA$^+$, are nonsynchronous. Note that the TS structures for the endergonic hydride transfer reactions from alcohols to Xn$^+$ and PhXn$^+$ have been found to have non-perfectly synchronized rehybridizations between donor and acceptor as well.[23,30] All these suggest that this class of hydride transfer reactions involving NADH/NAD$^+$ models would be nonclassical. This is consistent with the findings from NADH/NAD$^+$ model systems by other groups using different ways of analysis.[41-44]

Analysis within a H-tunneling mechanism from inflated α-2° KIEs: Solution (less inflated) vs. enzymes (more inflated)

A close examination of the α-2° H/D KIE value (1.09) at C-9 of MAH in its reaction with Tr$^+$ (close to the EIE (1.109)) shows that the KIE is inflated relative to the exergonic reaction whose KIE is supposed to be closer to unity for an sp^3 to sp^2 process. Meanwhile, the α-2° KIEs at the C-9 position of the MA$^+$ in its reactions with various donors are also inflated (1.00 to 1.02 in Table 2), which is supposed to be inverse and closer to unity for an exergonic sp^2 to sp^3 process. We have reported that in some of these reactions, for example, DMAH/Tr$^+$ and BNAH/DMA$^+$, the β-2° KIEs at the 9-CH$_3$/CD$_3$ groups of DMAH/DMA$^+$ are 1° isotope dependent (*i.e.*, breakdown of the Geometric Mean[45]).[31] Also, the temperature dependence of 1° KIEs in the reactions are outside of the semiclassical limits.[23,31,41] All of these strongly suggest that the reactions take place by nonclassical H-tunneling mechanisms.

Thus far, the observed inflated 2° KIEs on NADH/NAD$^+$ have been explained by three tunneling models, (i) the traditional 1°/2°

H coupled motions model within a one-dimensional Bell-type tunneling mechanism,[9,12,14,32] (ii) the contemporary multidimensional H-tunneling model within the variational TS theory,[19,25] and (iii) the recent activated (Marcus-like) H-tunneling model.[21,22,30,46] Earlier, such findings were all explained using the 1°/2° coupled motions model. All of the relevant discussions were qualitative concerning whether the reactions follow a tunneling mechanism and have 1°/2° H coupled motions. The latter two contemporary H-tunneling models, however, allowed to fit the inflated 2° KIEs to obtain the TS structures with the hybridization information. For example, in the exergonic zinc benzyl alcoholate oxidation with NAD^+ mediated by a yeast alcohol dehydrogenase (*y*ADH), the observed 2° H/T KIE at the benzylic position of the alcohol is 1.33, equal to the EIE of 1.33 (for sp^3 to sp^2), whereas the 2° H/D KIE at the C-4 position of the NAD^+ is 1.08, which is also outside of the range from unity to EIE (0.89).[47] Roston and Kohen fitted the inflated KIEs to the Marcus-like H-tunneling model, and found a non-perfectly synchronized H-tunneling TS (or tunneling ready state (TRS)).[47] The hybridizations of the donor and acceptor C's were found to be $sp^{2.76}$ and $sp^{2.34}$, respectively. Gao, Truhlar and co-workers calculated the TS structure for the hydride transfer reaction from NADH to dihydrofolate (DHF) mediated by the *Escherichia coli* (*ec*DHFR), using the ensemble-averaged multidimensional H-tunneling model within the variational TS theory.[25,48] Calculation showed that the 2° H/D KIE at the C-4 of NADH equals to 1.13, which is exactly the same as the observed one.[32] When they "removed" the H-tunneling contribution, the KIE became 1.03 suggesting a reactant-like TS. In the TS structure, they found that the C-4 of NADH has an $sp^{2.68}$ hybridization which is also reactant-like. Interestingly, in the latter two reactions, the 2° KIEs on the donors are equal to the EIE, but in their tunneling TS's the hybridizations are closer to the sp^3 state of the reactant (relative to sp^2), which seems in accordance with Hammond's postulate for an exergonic reaction (benzyl alcoholate /NAD^+ or NADH/$DHFH^+$ (acid-catalyzed)). On the other hand, the hybridizations at acceptors are close to a TS where the transferring H is in the midway between the donor and acceptor (closer to $sp^{2.5}$). Importantly, by comparison of the hybridizations of the donor and acceptor moieties of these tunneling TS's, it was found that the rehybridizations on the donor *vs.* acceptor in both H-tunneling reactions are also non-perfectly synchronized, *i.e.* the sum of the hybridizations of the donor and acceptor is larger than 5.

According to above analysis on enzymes, since our reactions are exergonic, the hybridizations at C-9's of both MAH and MA^+ in the tunneling TS's are most likely reactant-like as well. Nevertheless, the less inflated α-2° KIEs on MAH/(D)MA^+ as well as our previously observed less inflated α-2° KIEs on Xn^+ than those on NADH/NAD^+ in enzymes, such as formate dehydrogenase, *y*ADH and *ec*DHFR, implicates that H-tunneling probability is most likely enhanced by these enzymes.

Imbalance between rehybridization and charge development from α-9- vs. 10-N-CH/D KIEs and Hammett analysis: Different behaviors in solution vs. in enzymes

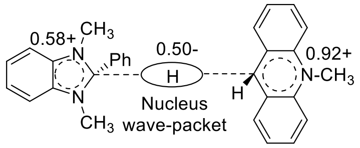
While the α-2° KIEs on the donor/acceptor C of the (D)MAH/(D)MA^+, after "removing" the H-tunneling effect, can provide information about the rehybridizations of the corresponding C's, the β-2° CH_3/CD_3 KIEs at the N position can provide information about the charge development on the structure containing N during the reaction, *i.e.*, can reflect the information about the electronic bond cleavage/formation, *i.e.*, extent of H-transfer (tunneling) in the tunneling ready TS. Note that unlike the α-2° KIEs, all the N-CH_3/CD_3 2° KIEs fall in between unity and the corresponding EIEs, implicating that tunneling would not have an extra effect on this remote 2° KIE. Therefore, its KIE/EIE comparison could be used to evaluate the charge development in the tunneling TS (see subsequent section for details). From the above analysis, the hybridization of the donor C is not closer to the product, whereas the N-CH_3/CD_3 2° KIEs are all closer or close to the EIE (Table 2). This appears to suggest that the rehybridization at the α-C lags behind its C-H bond breakage, *i.e.*, H-tunneling. Importantly, this is also supported by the Hammett correlations for the hydride transfer reactions from *G*-DMPBIH to MA^+ in acetonitrile, which gives rise to a Hammett constant (ρ) of -2.78 (Table 5 and Figure 1). The large Hammett slope is also consistent with a late tunneling ready TS in terms of the extent of loss of electron density from the DMPBIH donor during the reaction. We have previously reported a large similar Hammett ρ value of -2.66 for the hydride transfer reaction from para-substituted benzyl alcohols to $PhXn^+$ in acetonitrile.[23] In that reaction, the 2° KIE at the alcohol benzylic C also suggests that hybridization at the benzylic position in the TRS is reactant-like. The conclusion from that study is the same, *i.e.*, the rehybridization appears to lag behind the H-tunneling in solution.

In the *y*ADH enzyme, however, the hybridization of the benzyl alcohol donor at the tunneling ready TS is $sp^{2.76}$ which is reactant-like (see above), whereas the Hammett correlations for the hydride transfer from para-substituted benzyl alcohols to NAD^+ shows a Hammett ρ close to zero.[49,50] This suggests that rehybridization of the alcohol donor precedes the H-tunneling, which is opposite to the order observed from the model reactions. This latter comparison suggests that the enzyme may go through more changes in the donor and acceptor rehybridization process as part of the reorganization prior to H-tunneling, while in solution the H-tunneling occurs before the rehybridization of the substrate. This implicates a role of thermal motions of the *y*ADH enzyme in catalysis.

The electronic structure of the tunneling ready TS's from N-CH_3/CD_3 KIEs: Supported by Hammett correlations

It should be noted that above conclusion about the non-concerted rehybridization and charge development was drawn from the 2° KIE comparison at 9-CH/CD *vs.* N-CH_3/CD_3 positions of the donor moiety of the TS's only. A comparison of the N-CH_3/CD_3 KIEs *vs.* EIE for the donor (closer to EIE) *vs.* acceptor (closer to unity) in their reactions suggests that the donor moiety of the TS is product-like in terms of the charge distribution, whereas the acceptor moiety is reactant-like. Interestingly, this makes the whole TS carrying more than 1+ charge. Hammett correlations show that the reactions are much more sensitive to substituent effect on the donors than on the acceptors (Table 5), consistent with these KIE observations and further indicating this type of "unbalanced" charge development at the donor and acceptor in the TS. From the subsequent discussions, we will show that the transferring H indeed bears partial negative (-) charge being "hydridic" in character, which balances the "extra" (+) charge.

The fractional positive (+) charge (ζ) carried by the (D)MA and DMPBI moieties in the tunneling ready TS's can be calculated by comparison of the N-CH_3/CD_3 2° KIE with the corresponding EIE, *i.e.*, $\zeta = (1\text{-KIE})/(1\text{-EIE})$ for the donor and $\zeta = 1 - (1\text{-KIE})/(1\text{-EIE})$ for the acceptor. For example, for the reaction of DMPBIH with MA^+ (from the data in Tables 1 and 2), in the TRS the DMPBIH moiety carries 0.58+ charge (= (1-0.89)/(1-0.81)), whereas the MA moiety carries 0.92+ charge (= 1 - (1-1.01)/(1-1.13)). This leaves 0.50- charge borne by the transferring nucleus (Scheme 4). The Hammett constant on *G*-DMPBIH is -2.78 (Figure 1), seemingly consistent with the large amount of positive charge developed on the DMPBI moiety. The hydridic nature of the in-flight H in this type of reactions was also supported by the Hammett correlation studies of the hydride transfer reactions from 1-(para-substituted)benzyl-1,4-dihydronicotinamide (*G*-BNAH) to N-(para-substituted)benzyl-5-nitroisoquinolinium ion (*G*-BNIQ$^+$) in water containing 20% acetonitrile (v/v).[51] In that study, the BNAH moiety in the TS was found to bear 0.82+ charge and the BNIQ bears 0.62+ charge, indicating that the in-flight nucleus bears 0.44- charge. Importantly, in the latter example, the donor BNAH is also product-like in charge distribution, further supporting the possibility earlier discussed that the H transfer/tunneling precedes the rehybridization in the solution reactions. In the meantime, the smaller Hammett slope on the *G*-BNIQ$^+$ is also consistent with our observed close-to-unity 2° N-CH$_3$/CD$_3$ KIEs on (D)MA$^+$ in their reactions with different hydride donors.



Scheme 4. The electronic tunneling ready TS structure of the hydride transfer reaction from DMPBIH (left) to MA$^+$ (right) in acetonitrile; The nucleus in tunneling carries partial negative charge being "hydridic" in character.

In conclusion, the α-H/D 2° KIEs on the donor C and β-type N-CH/D 2° KIEs on the basic N in the NADH analogues, as well as those on the acceptor C and N$^+$ in the NAD$^+$ analogues in their respective hydride transfer reactions, in acetonitrile, were determined. Classically, the α-2° KIEs on the donor/acceptor C are resulted from the rehybridization, whereas those on N/N$^+$ resulted from the electron pair loss/gain. The corresponding 2° EIEs were computed to compare with the KIEs in attempts to examine the rehybridization and quantitative charge development in the TS on the reaction coordinate. Hammett correlations either constructed by us using the literature data or directly from literature were used to assist the electronic TS structure analysis. The results include (1) the α-2° KIEs on both the donor/acceptor C are inflated relative to the semiclassical predictions, but less inflated than those in the closely related enzymes; (2) the electronic structures of the donors in the TS's resemble product, whereas those of the acceptors resemble the reactant, indicating the electron density loss from the donors is more than its gain to the acceptors. The results, together with other observations such as the nonclassical temperature dependence of 1° KIE as well as the 1° isotope dependence of 2° KIEs, strongly support a H-tunneling mechanism in which the tunneling nucleus bears electron density being hydridic in character. In the tunneling ready TS's, the rehybridization appears to lag behind the H-tunneling on the donor side. The results and analyses were compared to those from the related enzymes. The H-tunneling appears to be less probable in our model reactions in both this work and the previous work using (Ph)Xn$^+$ as NAD$^+$ models. In *y*ADH, it was found that the rehybridization precedes the H-tunneling. This comparison suggests that the enzyme may lead to more changes in the donor and acceptor hybridization as part of the reorganization prior to H-tunneling, whereas in solution the H-tunneling occurs before the rehybridization of the substrate. Furthermore, the β-type N-CH/CD 2° KIEs on the N reaction center can be used to study the electronic structures of the relevant reactions when compared with the corresponding EIE. This can be a new approach that can play the same role as the Hammett correlation studies. The advantage is that it does not require synthesis of complex substrates that contain the bulky substituted aromatic groups and are thus less applicable for enzyme studies.

Experimental

General procedures

Syntheses of the following compounds were previously reported from our group: 10-methylacridiniumion (MA$^+$BF$_4^-$) and its 9-D substituted analogue (MA(9-D)$^+$), 9,10-dimethylacrdinium ion (DMA$^+$BF$_4^-$), 10-methylacridine (MAH), 9,10-dimethylacridine (DMAH), 9-phenylxanthylium ion (PhXn$^+$BF$_4^-$), 1-benzyl-1,4-dihydronicotinamide (BNAH).[23,31,52] The D content in MA(9-D)$^+$ is ~ 95% by NMR (see footnote b in Table 2 for discussion and *SI* for the NMR spectrum (Figure S6)). The commercially available tropylium ion (Tr$^+$BF$_4^-$) was recrystallized twice from acetonitrile/ether mixture before use. The 10-(deuterated-methyl)acridine (MAH-10-CD$_3$) and the 9-methyl-10-(deuterated-methyl)acridine (DMAH-10-CD$_3$) were synthesized from the reduction of the 10-(deuterated-methyl)acrdinium iodide (MA$^+$-10-CD$_3$) and 9-methyl-10-(deuterated-methyl)acridinium iodide (DMA$^+$-10-CD$_3$) by NaBH$_4$ in methanol following the same procedures to synthesize MAH and DMAH.[31,53] The latter two iodide salts were synthesized using the CD$_3$I following the same literature procedure for the synthesis of MA$^+$ and DMA$^+$ salts that used CH$_3$I.[31,53] The NMR spectra for the latter four 10-CD$_3$ compounds (Figures S7 to S10) show that the H content in these deuterated (10-CD$_3$) compounds was not detectable. The 1-(*p*-methylbenzyl)-1,4-dihydronicotinamide (CH$_3$BNAH) was synthesized according to a procedure described in literature.[41] The syntheses of the Hantzsch ester (diethyl 1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate, HEH) were done by a three components coupling reaction of ethyl acetoacetate, ammonia and formaldehyde following the literature procedure.[54] The 1,2-dimehtyl-2-phenyl-1,2-dihydrobenzimidazole (DMPBIH) and its 1,2-di(trideuterated methyl) analogue were prepared by reduction of the 1,3-dimethyl(or deuterated dimethyl)-2-phenyl-benzimidazolium iodide with NaBH$_4$, following the same procedure as in literature.[55] The precursor salt for the latter, 1,3-dimethyl(or deuterated dimethyl)-2-phenyl-benzimidazolium iodide, was synthesized from the reaction of 2-phenylbenzimidazole with CH$_3$I or CD$_3$I in methanol in the presence of NaOH in a sealed tube at 120 °C for 10 hours, according to the method described in literature.[55] All compounds synthesized were usually recrystallized three times before kinetic measurements. The ^{1}H NMRs for CH$_3$BNAH, HEH, DMPBIH as well as DMPBIH-1,3-N,N-2CD$_3$ are in *SI* (Figures S11 to S14). The D content in DMPBIH-1,3-N,N-2CD$_3$ was found to be 85% (by NMR in Figure S14). We did not look for other method to obtain the pure isotopic compound, instead, we corrected the kinetics to derive the 2° KIEs (See *SI*).

The HPLC grade acetonitrile was carefully redistilled twice under nitrogen, with the presence of KMnO$_4$/K$_2$CO$_3$ (to remove the reducing impurity) and P$_2$O$_5$ (to remove water) in order, for kinetic measurements.

Kinetic procedures

All kinetics were determined in acetonitrile under pseudo-first-order conditions at certain temperatures. Most measurements were conducted on an UV-Vis spectrophotometer with a cuvette connected to a rapid stopped-flow mixer (a Hi-Tech Scientific SFA-20 fast kinetic determination kit) or on a SF-61DX2 Hi-Tech KinetAsyst double-mixing stopped-flow instrument. Slow kinetics were determined with a thermostated cuvette in an Agilent 8453 UV-Vis spectrophotometer. The kinetics were determined under the pseudo-first-order conditions to give pseudo-first order rate constants (k^{pfo}) (R^2 are in the range of 0.9990 - 1.0000). The effect of substrate concentrations on k^{pfo} for each reaction was examined and the reactions follow the second-order rate law. This is consistent with the literature.[37-40] The 2° KIE was calculated using the second-order rate constants (k's). The measurements of the kinetics of the reactions of the two isotopologues for KIE derivation (= k_H/k_D) were done on the same day (back to back) using the same batch of distilled acetonitrile solvent (4-6 kinetic runs each) and were repeated at least two more times on different days (apart from months or even years for some systems, see *SI*). We also often used different batches of substrates in the determination of each KIE in order to test the effect of possible different impurity on the KIE measurements, and we did not find apparent differences. All the kinetic data are summarized in Tables S1 to S14 in *SI.* Careful measurements were done and standard deviations are relatively small.

The kinetics for the reactions of MAH and DMAH with Tr^+ (large excess) in acetonitrile has been reported in our previous publication.[31] They were determined by spectroscopically monitoring the formation of the $(D)MA^+$ at 436 nm. Here in this work, we determined the 2° N-CH_3/CD_3 KIEs of the reactions of (D)MAH *vs.* (D)MAH-10-CD_3 with Tr^+ following the same procedure. The k^{pfo}'s were determined by plotting $\ln(A_\infty - A)$ *vs. t*. The second-order rate constant $k = k^{pfo}/[Tr^+]$. Therefore, KIE = $k_H/k_D = k^{pfo}_H/k^{pfo}_D$. (We used the same Tr^+ solution to react with the CH_3 and CD_3 substrates so that the $[Tr^+]$ is canceled in the KIE calculation for these systems using k^{pfo}_H/k^{pfo}_D.)

Kinetics for the reaction of $(D)MA^+$, $MA(9\text{-}D)^+$, $(D)MA(10\text{-}CD_3)^+$ with BNAH, CH_3BNAH, HEH and DMPBIH were determined by following the decay of the cations at 436 nm due to MA^+ and DMA^+. The H-donors are in larger excess. The kinetic measurement of the BNAH reaction has been reported.[31] The exemplified kinetic scans for the reaction of HEH and DMPBIH are shown in Figures S3 and S4 in *SI.* The obtained $-\ln(A - A_\infty)$ *vs. t* data gave the k^{pfo}. Second-order rate constants ($k = k^{pfo}$/[H-donor]) were used to calculate the 2° KIEs. Since the same H-donor solution was used to react with the $(D)MA^+$ and its deuterated analogue, the KIE = $k_H/k_D = k^{pfo}_H/k^{pfo}_D$.

The kinetics for the reactions of the DMAH and DMAH-10-CD_3 (large excess) with $PhXn^+$ were determined spectroscopically by following the absorbance (A) decay of the cation (the hydride acceptor) at 485 nm. A kinetic scan example is given in Figure S5 in *SI.* The pseudo-first-order rate constant (k^{pfo}) was derived from the slope of the $-\ln(A_t - A_\infty)$ *vs.* time t. The 2° KIE was calculated using the second-order rate constant k (= k^{pfo}/[DMAH]), namely,

$$KIE = \frac{k_H}{k_D} = \frac{k_H^{pfo}}{k_D^{pfo}} \times \frac{[DMAH - CD_3]}{[DMAH - CH_3]} \qquad (2)$$

In order to reduce the errors from the concentration terms, so as to determine the accurate KIE, the above concentration ratio was corrected by determining the absorbance (A) value of the diluted H/D isotopic donor kinetic solutions at λ_{max} = 286 nm (at least three times each. Thus the KIE was derived according to eqn (3) (assuming $\varepsilon_H = \varepsilon_D$),

$$KIE = \frac{k_H}{k_D} = \frac{k_H^{pfo}}{k_D^{pfo}} \times \frac{A_D}{A_H} \qquad (3)$$

In the derivation of the N,N-2CH_3/2CD_3 KIE on DMPBIH for their reactions with $(D)MA^+$, since the DMPBIH and DMPBH-1,3-2CD_3 are in large excess over $(D)MA^+$, eqn. (3) was also used. The $A_{H(313nm)}$ and $A_{D(313nm)}$ were determined for the DMPBIH and DMPBIH-1,3-2CD_3 solutions. Since the D content in the latter compound is 85%, the corrected KIEs was reported (see *SI*, Tables S4 and S5 and eqns S1 to S4)

Computational methods

All computations were run at the B3LYP/6-31+G* level of theory both in gas-phase or in acetonitrile medium using the polarizable continuum model (PCM). Geometries were optimized by Gaussian 09 and Spartan 14. Molecular frequencies were calculated using Gaussian 09 and the EIEs were calculated with the ISOEFF 07 software.[56] The higher level (MP2/cc-pVTZ) of calculation was examined and the results are consistent (See Table S15 in *SI*).

ASSOCIATED CONTENT

Supporting Information

Hammett correlations, the exemplified UV-Vis kinetic scans, detailed kinetic data, EIE comparisons in gas phase *vs.* in acetonitrile medium, atom coordinates and total energies of structures, ^1H NMR spectra. 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 interests.

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