

Origin of the Primary Kinetic Hydrogen Isotope Effects on *N*-dealkylation from *N*-alkylamine by Hemoproteins

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The atomic level reaction mechanism for *N*-dealkylation of *N*,*N*-dimethylaniline ($PhNMe_2$) by hemoproteins, in which a kinetic hydrogen isotope effect (KHIE) was observed, was investigated by quantum mechanical theoretical calculations. Using quantum mechanical theory, one can determine the primary KHIE by the difference in zero-point vibrational energies on normal vibrational modes which are related to the reaction coordinate of H (D or T) atom abstraction from a C–H (C–D or C–T) bond. On the basis of this idea, we theoretically determined the intramolecular primary KHIE for two cases of hydrogen atom abstraction in *N*-dealkylation of $PhNMe_2$; i.e., hydrogen atom abstraction from a neutral state and proton abstraction from a cation radical state. When the intramolecular primary KHIE obtained by theoretical calculation was compared with experimental results of the intramolecular KHIE on *N*-dealkylation of $PhNMe_2$, which was catalyzed by some hemoproteins, it was found that four experimental values can be interpreted only from the primary KHIE. However, many other experimental values can not be interpreted only from the primary KHIE.

It is believed that the ultimate species in the mechanism of monooxygenation by cytochrome P-450 is $[Fe^{3+}–O\bullet]$.[1] This ultimate species is also known as compound I produced in the reaction mechanism by peroxidase, and it may cause hydrogen atom abstraction from a substrate. We have clarified the mechanism previously.[2] Many experimental studies[3–10] have shown that a kinetic hydrogen isotope effect (KHIE) is observed in hydrogen atom abstraction from a C–H bond of the substrate, by cytochrome P-450 and peroxidase. Because *N*-dealkylation of *N*-alkylamine also starts from C–H bond fission, many experiments on the KHIE were carried out to elucidate the reaction mechanism.[7–10] In the reaction, low KHIEs were observed in cytochrome P-450 and chloroperoxidase, whereas high KHIEs were observed in horseradish peroxidase and hemoglobin.[8–10] To explain the difference in the KHIEs, two major mechanisms of hydrogen atom abstraction in *N*-dealkylation, which is catalyzed by hemoproteins, have been proposed: (i) simple hydrogen atom abstraction from a substrate, and (ii) proton abstraction from a cation radical that is formed by one electron abstraction from a substrate.[8–11] In the present study, values of the intramolecular primary KHIE on *N*-dealkylation of *N*,*N*-dimethylaniline ($PhNMe_2$) by hemoproteins were determined by quantum mechanical theoretical calculation, and these values were compared with experimental values to verify the validity of these two proposed mechanisms. The results showed that four experimental results can be interpreted only from the primary KHIE.

As for organic chemical reactions, some studies have already been carried out by Swain et al.[12] and Streitwieser et al.[13] Swain et al. showed that an equation that connects primary kinetic deuterium and tritium isotope effects, called the Swain–Schaad equation (Eq. 1), could explain experimental KHIEs at 0–100 °C.[12]

$$K_H/K_T = (K_H/K_D)^{1.442}. \quad (1)$$

When they derived this equation, they used a model in which hydrogen isotopes were bonded with a wall of infinite mass. On the other hand, Streitwieser et al. derived the same equation by using a ^{12}C–H (D or T) binding model and showed that the exponent was 1.427.[13] In the case of an enzyme reaction, Grant et al. experimentally measured KHIEs in bovine serum amine oxidase (BSAO)-catalyzed oxidation of benzylamine and calculated the exponent.[14] In the present study, we derived Eq. 1 for hemoprotein-catalyzed *N*-dealkylation of $PhNMe_2$ by quantum mechanical calculation considering the full structure of $PhNMe_2$, and we showed that the exponent of Eq. 1, 1.432, was between the values of Swain et al. and Streitwieser et al.

Because of the small mass of a hydrogen atom, many studies have considered the tunneling effect for the KHIE.[14–21] Saunders et al. argued that the tunneling effect was important in the model reaction $CCH_2CH_2Cl + OH^- \rightarrow CCH{=}CH_2 + Cl^-$.[15] Klinman's group experimentally determined the KHIE for the catalytic oxidation of benzyl alcohol by yeast alcohol dehydrogenase (YADH), and they compared the observed K_H/K_T values with K_H/K_T values calculated from the Swain-Schaad equation. Because the experimental values were much greater than the calculated values in both cases of the primary and the secondary KHIEs, they argued that hydrogen tunneling occurred in this enzymatic reaction.[16,17] They showed that the calculated KHIE values close to the experimental ones were reproduced by considering the tunneling effect in the transition state and its environs[18] using the BEBOVIB IV program.[22] They also experimentally measured KHIEs in BSAO-catalyzed oxidation of benzylamine, and they argued from the results of very large primary KHIE (K_H/K_T) values and anomalously low Arrhenius preexponential factor ratios (A_H/A_T and A_D/A_T) that both protium and deuterium underwent significant

tunneling in the course of substrate oxidation.[14,17] Antonious et al. proposed two different tunneling models to explain this primary KHIE: (1) proton tunneling from the ground state, and (2) both over-the-barrier transfer and tunneling from excited states. It has been reported that the difference in the manner of interaction between a substrate and [Fe^{3+}–O•] at an active site[9] and the rotation around an amide bond of a substrate at an active site[23] influences the KHIEs on hemoprotein-catalyzed *N*-demethylation of *N*-alkylamine and *N*-alkylamide. Since KHIE values obtained from experimental data of hemoprotein-catalyzed demethylation are both larger and smaller than those predicted from the Swain-Schaad equation, it is considered that the tunneling effect and other sources have complex effects on the KHIE. We, however, only discuss the effect of zero-point vibration in this paper.

Theoretical Consideration

It is thought that in the saddle point on hydrogen atom abstraction from a C–H bond, atoms that relatively and remarkably change their position coordinates localize at the C–H bond in order to react and that the relative configuration among other atoms is not greatly different from that in the initial state of the reaction. Thus, the activation energy in the reaction is the potential energy difference between the saddle point located on the reaction coordinate of the reaction and the normal vibration in the initial state of the reaction, which is related to the reaction coordinate. However, we determine the potential energy hypersurface using the mass-weighted coordination system in order to fix the mass of a representative point in the reaction system. In the reaction coordinate of hydrogen abstraction from a C–H bond on this potential energy hypersurface, the potential energy values of the saddle point are the same in all cases of C–H, C–D (Deuterium), and C–T (Tritium) bonds. However, the activation energy changes because zero-point vibrational energy in each case differs in the initial state of the reaction.[24] For this reason, the intramolecular primary KHIE on *N*-dealkylation of $PhNMe_2$ can be determined by the difference between the zero-point vibrational energies of the normal vibrations, each of which is related to the reaction coordinate of the hydrogen (or the isotope) atom abstraction from a C–H (C–D or C–T) bond. If the zero-point vibrational energies of these normal vibrations are E_H, E_D, and E_T, respectively, and the activation energy in hydrogen atom abstraction is E_a, the rate constants K_H, K_D, and K_T are

$$K_H = A_H \exp(-(E_a - E_H)/RT) \quad (2)$$

$$K_D = A_D \exp(-(E_a - E_D)/RT) \quad (3)$$

$$K_T = A_T \exp(-(E_a - E_T)/RT) \quad (4)$$

from the Arrhenius equation, where A_H, A_D, and A_T are preexponential factors in H, D, and T atom abstractions, respectively, and R is a gas constant and T is temperature.

Schneider et al. calculated the ratio of the Arrhenius preexponential factor $A_Q = A_H/A_D$ for the primary KHIE in the absence of quantum mechanical tunneling, and they estimated the ratio to be $A_Q \geq 0.5$.[25] On the other hand, Klinman's group reported that the ratio of the Arrhenius preexponential factor for light (L_1) and heavy (L_2) isotopes was $A_{L1}/A_{L2} \approx 1$ in case of classical behavior (no tunneling).[20,21] In this study, since the tunneling effect was not considered, we assumed that

$$A_H = A_D = A_T. \quad (5)$$

Then, KHIEs are

$$K_H/K_D = \exp((E_H - E_D)/RT) \quad (6)$$

$$K_H/K_T = \exp((E_H - E_T)/RT). \quad (7)$$

With regard to this isotope effect, two cases were investigated; i.e., hydrogen (or the isotope) atom abstraction from an *N*-methyl group of $PhNMe_2$ and a proton (or the isotope ion) abstraction from an *N*-methyl group of a $PhNMe_2$ cation radical. Two T values (298.15 K (25 °C) and 310.15 K (37 °C)) were considered because these values had been used in an experimental study.[9]

Method

Density functional theory (DFT) calculations were performed for the structures of $PhNMe_2$ and its cation radical. The basis set used was 6-31G**. The exchange functional was Becke's three parameter functional,[26] and the correlation functional was Lee–Yang–Parr's formula.[27] The minimum point on the potential energy hypersurface was obtained by geometry optimization, allowing all atoms to move freely to find a stable structure of the molecule. Normal frequency analysis was performed for the stable structure to confirm that all the frequencies corresponding to the normal vibration modes of the molecule were real numbers; i.e., the structure was the correctly energy-minimized structure. In the above optimized structures, all hydrogen atoms belonging to one of the two methyl groups were substituted with deuterium or tritium ([N–^{1}H$_3$,N–^{2}H$_3$]$PhNMe_2$, [N–^{1}H$_3$,N–^{3}H$_3$]$PhNMe_2$ and their cation radicals), and normal frequency analysis was performed on these isotope-incorporated molecules. A vibrational mode localizing in stretching motion which is related to the bond fission of a C–H, C–D, or C–T bond was selected for each case, and then their frequency (ν) was estimated. The computational program used was Gaussian 98.[28]

The zero-point vibrational energy of the vibration was calculated by the equation $h\nu/2$, where h is the Planck constant. Further, the primary KHIE was evaluated according to the procedure described in the previous section.

Results

The most stable structure of $PhNMe_2$ is shown in Fig. 1a. A comparison of the upper and lower figures shows that the structure is symmetric to a plane including N, C(3), C(4), and H(7) atoms. Normal frequency analysis of the structure was performed, and the results showed that all of the vibrational numbers for each vibrational mode were real numbers; i.e., the structure existed in a minimum point on the potential energy hypersurface.

From the structure, we elucidated hydrogen atom abstraction from a C–H bond. Although there were six hydrogen atoms in the two *N*-methyl groups, the case of H(1) atom ab-

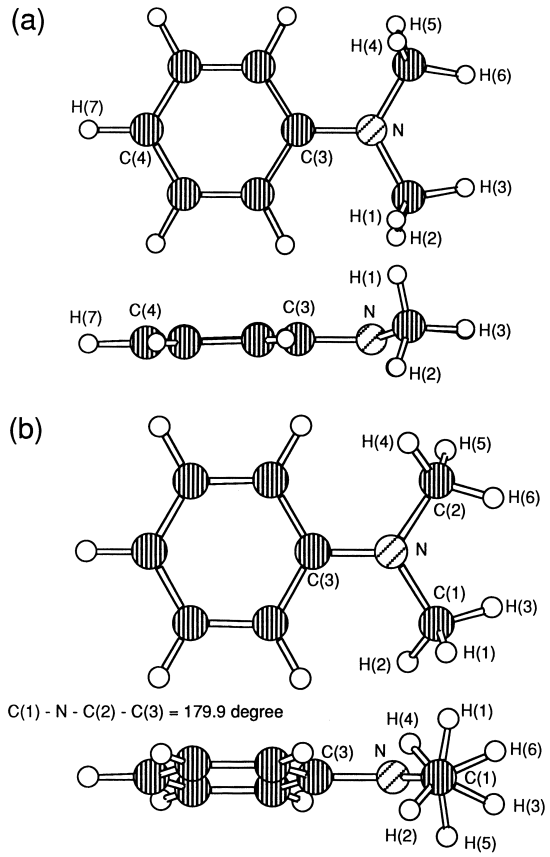

Fig. 1. The most stable structures for (a) neutral species and (b) cation radical of *N*,*N*-dimethylaniline, which were obtained by DFT method.

straction was considered. To investigate the intramolecular primary KHIE, normal frequency analysis was performed on two cases: one was H(4) to H(6)-deuterated $PhNMe_2$ to consider H(1) atom abstraction, and the other was H(1) to H(3)-deuterated $PhNMe_2$ to consider D(1) atom abstraction. Then, the vibrational mode of the normal frequency which was related to the reaction coordinate of H(1) or D(1) atom abstraction from an *N*-methyl group was investigated. The results are shown in Fig. 2. Vibrational numbers of the normal frequencies which were related to the reaction coordinates of H(1) and D(1) atom abstractions were 2986.3 cm^{-1} and 2153.9 cm^{-1},

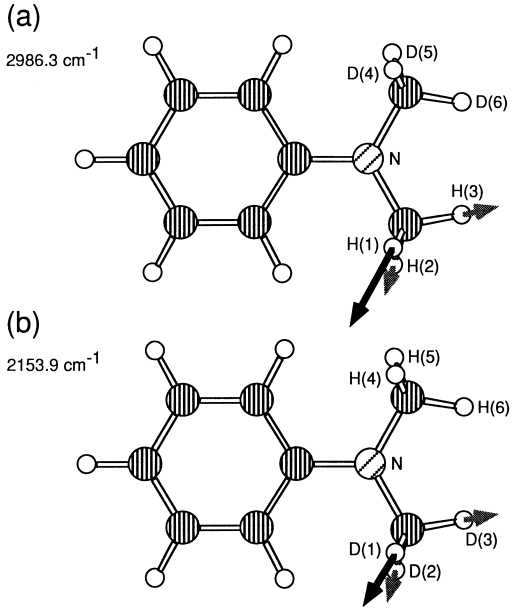

Fig. 2. (a) Normal vibrational mode and its frequency which are related to H(1) atom abstraction from an *N*-methyl group of $[N–^1H_3,N–^2H_3]PhNMe_2$. (b) The same for D(1) atom.

respectively. Using these values, the intramolecular primary KHIEs were calculated by the process described in "Theoretical consideration" section. As shown in Table 1, the K_H/K_D values were 7.451 in 298.15 K and 6.894 in 310.15 K. The same process was performed for H(4) to H(6)-tritiated $PhNMe_2$ and H(1) to H(3)-tritiated $PhNMe_2$. The vibrational modes which are related to the reaction coordinates of H(1) and T(1) atom abstractions from *N*-methyl groups are shown in Fig. 3. In this case, vibrational numbers are 2986.3 cm^{-1} and 1794.4 cm^{-1}, respectively. Using these values, the intramolecular primary KHIEs were calculated by the process described in "Theoretical consideration" section. The K_H/K_T values were 17.742 in 298.15 K and 15.873 in 310.15 K (Table 1).

We elucidated the structure of not only $PhNMe_2$ but also that of the $PhNMe_2$ cation radical. The most stable structure of the cation radical is shown in Fig. 1b. The nitrogen atom and three carbon atoms, C(1) to C(3), binding to the nitrogen atom, are on the same plane. As shown in the lower Figure of Fig.

Table 1. Zero-Point Vibrational Energies (kJ/mol) for the Normal Vibrational Modes Related to the H, D, and T Atom Abstraction Reactions from *N*-Methyl Groups, Which Were Obtained by Theoretical Calculations, and the Intramolecular KHIEs Estimated by Eqs. 6 and 7 Using the Zero-Point Vibrational Energy Values

Molecule	Zero point vibrational energy/kJ mol^{-1}			Intramolecular kinetic hydrogen isotope effect			
				K_H/K_D		K_H/K_T	
	H	D	T	298.15 K	310.15 K	298.15 K	310.15 K
$[N–^1H_3,N–^2H_3]PhNMe_2$	17.862	12.883	—	7.451	6.894	—	—
$[N–^1H_3,N–^3H_3]PhNMe_2$	17.862	—	10.733	—	—	17.742	15.873
$[N–^1H_3,N–^2H_3]PhNMe_2$ cation radical	18.294	13.151	—	7.960	7.346	—	—
$[N–^1H_3,N–^3H_3]PhNMe_2$ cation radical	18.294	—	10.928	—	—	19.518	17.399

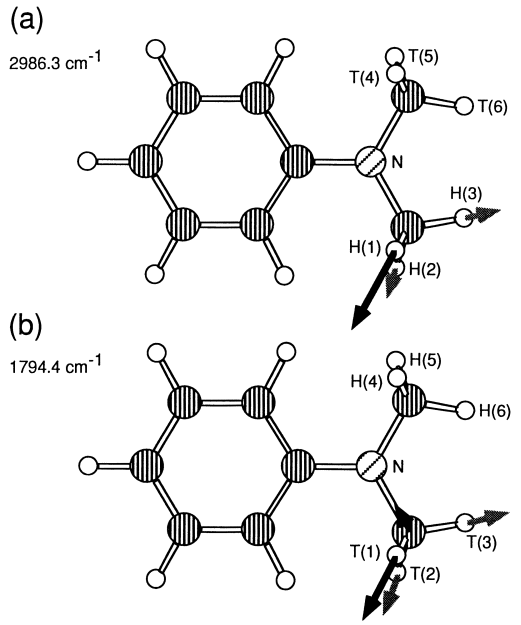

Fig. 3. (a) Normal vibrational mode and its frequency which are related to H(1) atom abstraction from an *N*-methyl group of $[N–^1H_3,N–^3H_3]PhNMe_2$. (b) The same for T(1) atom.

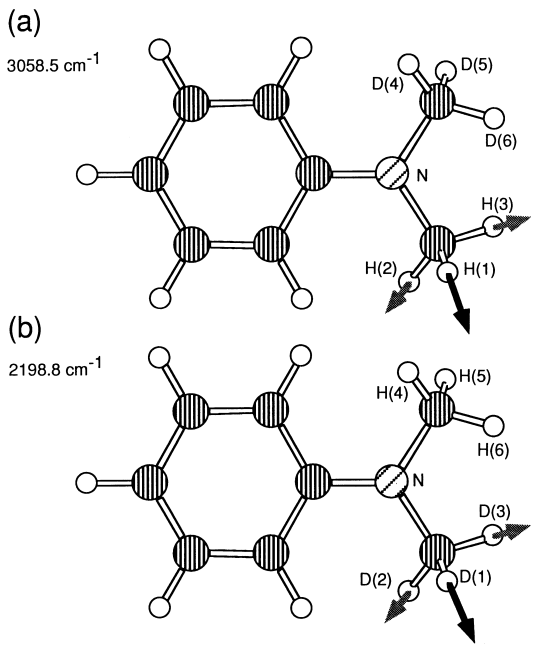

Fig. 4. (a) Normal vibrational mode and its frequency which are related to $H^+(1)$ abstraction from an *N*-methyl group of $[N–^1H_3,N–^2H_3]PhNMe_2$ cation radical. (b) The same for $D^+(1)$.

1b, imposing the C(1) atom on C(2) atom, it was found that H(1) to H(6) atoms have a gauche-type conformation. Normal frequency analysis of the structure was performed, and the results showed that all of the vibrational numbers for each vibrational mode were real numbers; i.e., the structure existed in a minimum point on the potential energy hypersurface.

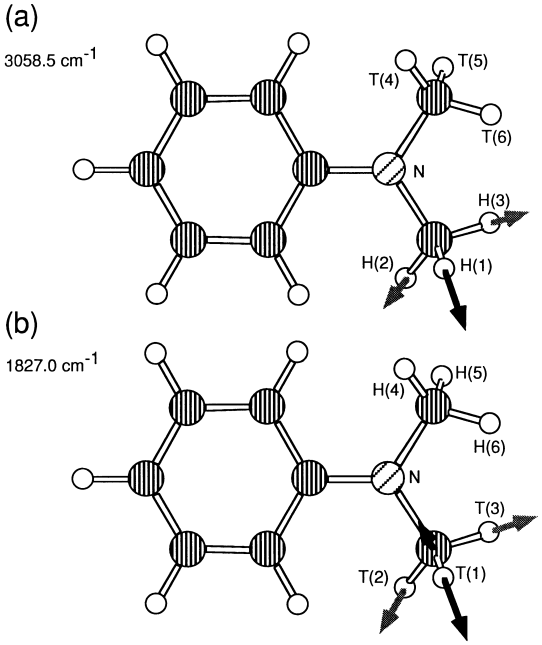

Fig. 5. (a) Normal vibrational mode and its frequency which are related to $H^+(1)$ abstraction from an *N*-methyl group of $[N–^1H_3,N–^3H_3]PhNMe_2$ cation radical. (b) The same for $T^+(1)$.

From the structure, we elucidated proton abstraction from a C–H bond. Although there were six hydrogen atoms in the two *N*-methyl groups, the case of $H^+(1)$ abstraction was considered. To investigate the intramolecular primary KHIE, normal frequency analysis was performed on two cases: one was H(4) to H(6)-deuterated $PhNMe_2$ cation radical to consider $H^+(1)$ abstraction, and the other was H(1) to H(3)-deuterated $PhNMe_2$ cation radical to consider $D^+(1)$ atom abstraction. Then, the vibrational mode of the normal frequency which was related to the reaction coordinate of $H^+(1)$ or $D^+(1)$ abstraction from an *N*-methyl group was investigated. The results are shown in Fig. 4. Vibrational numbers of the normal frequencies which were related to the reaction coordinates of $H^+(1)$ and $D^+(1)$ abstractions were 3058.5 cm^{-1} and 2198.8 cm^{-1}, respectively. Using these values, the intramolecular primary KHIEs were calculated by the process described in "Theoretical consideration" section. As shown in Table 1, the K_H/K_D values were 7.960 in 298.15 K and 7.346 in 310.15 K. The same process was performed for H(4) to H(6)-tritiated $PhNMe_2$ cation radical and H(1) to H(3)-tritiated $PhNMe_2$ cation radical. The vibrational modes which are related to the reaction coordinates of $H^+(1)$ and $T^+(1)$ abstractions from *N*-methyl groups are shown in Fig. 5. In this case, the vibrational numbers are 3058.5 cm^{-1} and 1827.0 cm^{-1}, respectively. Using these values, the intramolecular primary KHIEs were calculated by the process described in "Theoretical consideration" section. The K_H/K_T values were 19.518 in 298.15 K and 17.399 in 310.15 K (Table 1).

Discussion

Relation between K_H/K_D and K_H/K_T Values. As described previously, Swain et al. theoretically found the relation

between primary kinetic deuterium and tritium isotope effects (Eq. 1).[12] Using our results obtained by theoretical calculation, the following relations were obtained. In the case of hydrogen (or the isotope) atom abstraction from an *N*-methyl group of $PhNMe_2$, the equations are

$$K_H/K_T = (K_H/K_D)^{1.431990048} \approx (K_H/K_D)^{1.432} \quad (8)$$

at 298.15 K, and

$$K_H/K_T = (K_H/K_D)^{1.431962008} \approx (K_H/K_D)^{1.432} \quad (9)$$

at 310.15 K. In the case of proton (or the isotope ion) abstraction from an *N*-methyl group of a $PhNMe_2$ cation radical, the equations are

$$K_H/K_T = (K_H/K_D)^{1.432363854} \approx (K_H/K_D)^{1.432} \quad (10)$$

at 298.15 K, and

$$K_H/K_T = (K_H/K_D)^{1.432391853} \approx (K_H/K_D)^{1.432} \quad (11)$$

at 310.15 K. It was found that Eqs. 8 to 11 obtained by theoretical calculation agreed well with both Eq. 1 and the equation proposed by Streitwieser et al.[13] Both equations derived by Swain et al. and Streitwieser et al. assumed a primary KHIE. The KHIEs obtained in this study are primary KHIEs because the KHIE values were obtained from the frequencies of the normal vibrational modes localizing the hydrogen abstaction in the ground state. The values can reproduce both equations on the assumption that the Arrhenius preexponential factors for H, D, and T atoms are equal to each other. As described later, four samples of the hemoproteins can be explained only by the primary KHIE.

Comparison with Experimental Results. Okazaki et al. reported an intramolecular KHIE on *N*-dealkylation of $PhNMe_2$. They considered the tritium isotope effect as well as the deuterium isotope effect, and they investigated the effects of several hemoproteins, including cytochrome P-450 and horseradish peroxidase.[9] As described in the following part, it was found that four samples of the hemoproteins could be explained by only the primary KHIE that was elucidated in this study. The experimental values are shown in Table 2. Temperatures during the experiment were 310.15 K (37 °C) for cytochrome P-450 and hemoglobin, and 298.15 K (25 °C) for prostaglandin H synthase and horseradish peroxidase.

Northrop proposed an equation to calculate the intrinsic KHIE from two apparent KHIEs, $(V/K)_H/(V/K)_D$ and $(V/K)_H/(V/K)_T$.[29]

$$\frac{(V/K)_H(V/K)_D - 1}{(V/K)_H(V/K)_T - 1} = \frac{(K_H/K_D) - 1}{(K_H/K_T) - 1} \quad (12)$$

V is V_{max} value and K is K_m value. Since it is found from both sides of Eq. 12 that the expressions of both sides are the same forms and that $(V/K)_H/(V/K)_D$ and $(V/K)_H/(V/K)_T$ terms ($^D(V/K)$ and $^T(V/K)$ in Table 2, respectively) are related to (K_H/K_D) and (K_H/K_T) terms, respectively, we can compare the intramolecular KHIE obtained from experimental results with that obtained by our theoretical results. The values are shown in Table 2. The theoretical values of the hemoprotein are very different from the experimental values because of the different processes used to reach the values. Then, we tried to normalize the theoretical values with the experimental $^D(V/K)$ values,

Table 2. Comparison of the Intramolecular KHIE between Experimental Measurements[9] and the Present Theoretical Work. The Experimental Values of These Four Cases Can Be Interpreted Only from the Primary KHIE

System[a]	Experimental results[a]	Theoretical results[f]	
		neutral[d]	cation[e]
	$^D(V/K)$[b]	K_H/K_D	K_H/K_D
	$^T(V/K)$[c]	K_H/K_T	K_H/K_T
P450 2B1, reductase, NADPH	1.4	6.894 (1.4)	7.346 (1.4)
(310.15 K)	3.4	15.873 (3.2)	17.399 (3.3)
Prostaglandin H synthase, H_2O_2	3.5	7.451 (3.5)	7.960 (3.5)
(298.15 K)	8.1	17.742 (8.3)	19.518 (8.6)
Hemoglobin, C_2H_5OOH	5.2	6.894 (5.2)	7.346 (5.2)
(310.15 K)	13.6	15.873 (12.0)	17.399 (12.3)
Horseradish peroxidase, C_2H_5OOH	5.3	7.451 (5.3)	7.960 (5.3)
(298.15 K)	12.7	17.742 (12.6)	19.518 (13.0)

a) Ref. 9. b) $^D(V/K) = (V/K)_H/(V/K)_D$. From Ref. 9. c) $^T(V/K) = (V/K)_H/(V/K)_T$. From Ref. 9. d) $PhNMe_2$. e) $PhNMe_2$ cation radical. f) Determined by the procedure described in "Theoretical consideration" section. Numerals in parentheses are relative values normalized by $^D(V/K)$ value obtained by experiments.

and the calculated values are shown in parentheses in Table 2. All of the values agree well with the experimental ones. The KHIE obtained experimentally usually contains different factors from the KHIE occurring due to hydrogen (or the isotope) atom or proton (or the isotope cation) abstraction. However, it was found by comparison with the theoretical values that the primary KHIE caused by hydrogen (or the isotope) atom or proton (or the isotope ion) abstraction itself is reflected in the experimental values of Table 2. From the experimental values, the kinetic isotope effect obtained by standardizing hydrogen atoms differs for each hemoprotein, whereas that obtained by standardizing deuterium atoms does not greatly differ; i.e., $^{D}(V/K)/^{T}(V/K)$ (or K_D/K_T) is about two in both the experimental and theoretical results.

Table 2 shows the hemoproteins whose KHIEs we could explain only by using the primary KHIE. However, many experimental values[8,9] can not always be interpreted only by the primary KHIE, because they may be modified by some other factors; e.g., difference in the manner of the interaction between a substrate and [Fe^{3+}–O•] at an active site,[9] rotation around an amide bond of a substrate at an active site,[23] tunneling effect and secondary KHIE.

Miwa et al. argued on the basis of intramolecular KHIEs on *N*-dealkylation of several hemoproteins that hydrogen atom abstraction from a substrate occurred in some hemoproteins (e.g., horseradish peroxidase) that yielded high KHIEs, while deprotonation from a substrate occurred in hemoproteins (cytochrome P-450 and chloroperoxidase) that yielded low KHIEs.[8] Okazaki et al. also investigated intramolecular KHIEs on *N*-dealkylation of several hemoproteins. Based on their results, they claimed that cytochrome P-450 and chloroperoxidase catalyzed deprotonation from an ammoniumyl radical (substrate) after one-electron abstraction, and that horseradish peroxidase, prostaglandin H synthase, and hemoglobin catalyzed only one-electron abstraction and that is why high KHIEs were observed in the hemoproteins.[9] As shown in Table 2, however, it was found that the normalized theoretical values are not greatly different between a neutral case ($PhNMe_2$) and a cation case ($PhNMe_2$ cation radical). This results suggests that we can not judge only from measurement of KHIEs which mechanism occurs in hemoprotein-catalyzed hydrogen atom abstraction in *N*-dealkylation, hydrogen atom abstraction from a substrate or proton abstraction after cation radical formation by one-electron abstraction from a substrate.

Conclusion

Considering *N*-dealkylation of $PhNMe_2$ as an example, the mechanism underlying the intramolecular KHIE, and the relation between primary kinetic deuterium and tritium isotope effects were investigated by quantum mechanical theoretical calculations, considering a full structure of $PhNMe_2$. The intramolecular primary KHIE determined by the theoretical calculations was compared with the intramolecular KHIE of hemoproteins determined by experiments. It was found that the experimental data $^{T}(V/K)$ were in good agreement with the theoretical data (K_H/K_T), standardizing experimental data $^{D}(V/K)$ and that four cases could be interpreted only from primary KHIEs. An equation that connected primary kinetic deuterium and tritium isotope effects, which was determined by quantum mechanical theoretical calculation in this study, considering a full structure of $PhNMe_2$, was practically the same as both the Swain–Schaad equation obtained by using a model in which H, D, or T atoms bonded with a wall of infinite mass and Streitwieser's equation which used a ^{12}C–H (D or T) binding model. Although two major mechanisms for hydrogen atom abstaction from $PhNMe_2$ which starts with *N*-dealkylation catalyzed by hemoproteins have been proposed, i.e., (i) hydrogen atom abstraction from a neutral state, and (ii) proton abstraction from a cation radical state, it was concluded that we could not judge which reaction occurred only from the KHIE values.

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