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Kinetics of the Hydride Reduction of an NAD^+ Analogue by Isopropyl Alcohol in Aqueous and Acetonitrile Solutions: Solvent Effects, Deuterium Isotope Effects, and Mechanism

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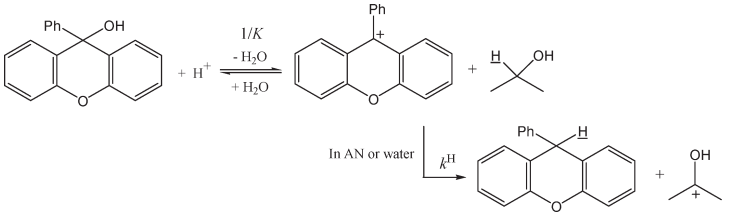

The rate constants of the hydride-transfer reactions from isopropyl alcohol (*i*-PrOH) to an NAD^+ model, 9-phenylxanthylium ion ($PhXn^+$), in acetonitrile (AN) and in water containing AN (80% H_2O/20% AN) were determined over a temperature range from 36 to 67 °C. The reactions follow second-order rate laws. In the latter solution, formation of the water adduct of $PhXn^+$ was observed as a side-equilibrium (K). The observed inverse solvent kinetic isotope effect ($k_{H_2O}{}^{obs}/k_{D_2O}{}^{obs} = 0.54$), the larger than unity equilibrium isotope effect ($K(H_2O)/K(D_2O) = 2.69$), and the results of acid effect on the observed rate constants of the reactions are consistent with the "side-equilibrium mechanism". Kinetic isotope effects at all three H/D positions of *i*-PrOH for the net hydride-transfer process were determined in both solutions at 60 °C: $KIE_{\alpha\text{-}D}{}^H = 3.2$(AN), 3.2($H_2O$); $KIE_{\beta\text{-}D6}{}^H = 1.05$(AN), 1.16($H_2O$); $KIE_{OD}{}^H = 1.08$(AN), 1.04(H_2O). These KIE values are consistent with the presence of the positively charged alcohol moiety in the transition state (TS) for cleavage of the α-C−H bond, the delocalization of the positive charge over the α-C−OH group, and the stepwise hydride and proton transfer processes. Comparison of the activation parameters for the reactions in the two solvent systems as well as those in the *i*-PrOH/AN (1:1 v/v) reported earlier suggests that the AN medium promotes the reaction by activating the ground-state alcohol reactant through weak interactions with the electron pairs on alcohol O, while water and parent alcohol media facilitate the reaction by H-bonding stabilization of the alcohol moiety of the TS. Results suggest that in the alcohol dehydrogenases without a Zn(II) cofactor in the active sites alcohols would be oxidized via hydride transfer to NAD^+ coenzyme followed by the rapid deprotonation to the nearby basic species in the active site of the enzymes.

Introduction

Alcohols in living organisms are metabolized to carbonyl compounds via oxidation by NAD^+ coenzyme mediated with alcohol dehydrogenases. The chemistry of the biological reactions involves hydride transfer from the α-C of the alcohol to the 4-C of the NAD^+ pyridinium ring. Much of the mechanistic understanding of the transformations has come from determinations of the reaction kinetics and study of the effects of the relevant factors on the kinetics.[1−4] Conventional kinetic investigations have, however, often been greatly challenged since the kinetic data determined usually do not completely correspond with the inherent hydride transfer processes due to the complexity of the enzymatic reactions.[5−7] Although various approaches have been developed in order to kinetically unmask the chemistry of the enzymatic reactions, it has been a great challenge to

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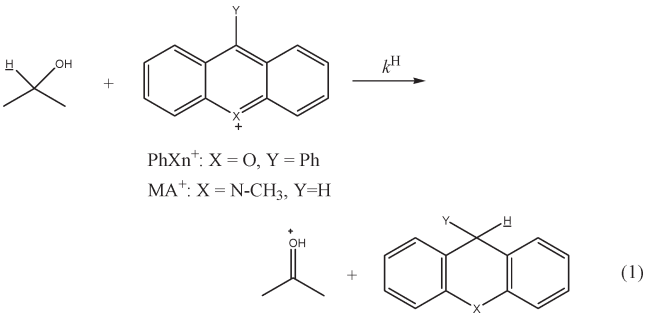
correctly extract the kinetics of these hydride-transfer steps for the derivation of the mechanistic information including the structure of the important transition state (TS).[8−10]

Studies of the kinetics of relatively simple non-enzymatic model reactions have been applied in order to provide useful insights into the mechanism of the biological enzymatic reactions. The corresponding investigations for the alcohol dehydrogenases have, however, not generally been successful[11−15] until very recently when we reported the thorough kinetic studies of hydride-transfer reactions from isopropyl alcohol (*i*-PrOH) to the two NAD^+ analogues 9-phenylxanthylium ion ($PhXn^+$) and 10-methylacridinium ion (MA^+), in the parent alcohol medium containing various amounts of acetonitrile (AN) or a small amount of water (eq 1).[16,17] The derived rate constants for the hydride transfers (k^H) together with the observed deuterium kinetic isotope effects (KIE) at all three H/D positions in *i*-PrOH were used to elucidate the hydride transfer TS structure and the H-bonding stabilization effect on the TS.[17] These TS structural features bear some similarities to those of the hydride-transfer processes of the alcohol dehydrogenase reactions.

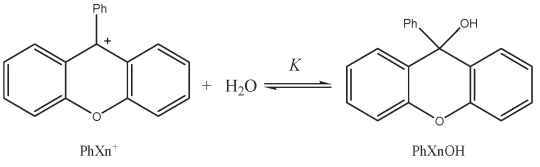


(1)

Understanding the factors affecting the rates of the non-enzymatic reactions and the stabilities of the corresponding TSs is expected to illuminate the nature of the enzymatic catalysis. In this paper, we will explore how changes in environments/reaction media affect the kinetics of the hydride-transfer reaction from *i*-PrOH to $PhXn^+$ and the nature of its TS. We have reported the kinetic study of the reaction in the parent alcohol medium containing AN (1:1 v/v);[17] herein we will report the corresponding study in aqueous solution (80% H_2O/20% AN (v/v)) and in aprotic AN. Since *i*-PrOH concentrations used in this work were low, complications due to the aggregation of the alcohols that most likely exists in the *i*-PrOH/AN solvents are expected to be diminished or eliminated. The latter effect can significantly affect the kinetics of the reactions and hence the mechanistic information obtained. Studies of the effects of isotopic substitution at the three H/D positions of *i*-PrOH, of the acid concentration, of the alcohol concentration, and of the temperature on the kinetics of the reactions were carried out. The results were compared with those earlier obtained for the same reaction in mixed solvents (*i*-PrOH/AN) as well as a β-D KIE value for an alcohol dehydrogenase reaction found in literature. Reaction mechanisms including the structures of the TSs were proposed.

Results

Hydration Equilibrium of $PhXn^+$ and the Equilibrium Isotope Effect (EIE). In 80% H_2O/20% AN, $PhXn^+$ (counterion, BF_4^-) exists in equilibrium with its water adduct PhXnOH (eq 2). The equilibrium concentration of $PhXn^+$ ($[PhXn^+]_{eq}$) increases with increasing $[H^+]$ (= $[HBF_4]$). Since the observed rate constant (k^{obs}) of the hydride-transfer reaction between *i*-PrOH and $PhXn^+$ in aqueous solution depends on $[PhXn^+]_{eq}$, it is necessary to determine the equilibrium constant (K) in order to derive kinetic information on the net hydride-transfer step (k^H) from the observed kinetic results in this reaction medium. The relationships between k^{obs}, k^H, and K will be discussed in the following sections (see eq 4). The value of K in 80% H_2O/20% AN at 60 °C, defined as $K(H_2O) = [PhXnOH]_{eq}[H^+]_{eq}/[PhXn^+]_{eq}$, was determined to be equal to 0.214 ± 0.035 M. Note that the K value might be comparable to the K_{R^+} value for the $PhXn^+$ that was determined in pure acidic aqueous solutions at 25 °C. The K_{R^+} of 0.148 and 0.079 M have been reported by Arnett[18] and Feldman,[19] respectively. In order to understand the kinetic solvent isotope effect ($k^{obs}(H_2O)/k^{obs}(D_2O)$), K in 80% D_2O/20% AN at the same temperature was also determined ($K(D_2O) = 0.0797 \pm 0.0134$ M). The equilibrium isotope effect (EIE = $K(H_2O)/K(D_2O)$) was observed to be equal to 2.69. The larger than unity EIE value indicates that under the same conditions $[PhXn^+]_{eq}$ is higher in D_2O medium than in H_2O medium.



(2)

The observed EIE value is consistent with the known deuterium fractionation factor (φ) for deuterium between H_2O and H_3O^+ sites.[20,21] In aqueous system, φ is defined to be the D/H ratio in the OL^+ bond (L = H and D) divided by the D/H ratio in the OL bond, i.e., $\varphi = (D/H)_{OL^+}/(D/H)_{OL}$. A φ value of 0.69 has been determined for the hydronium ion in the aqueous system.[20,21] This value means that, in D_2O solvent at room temperature, D has only 69% preference as compared to H in H_2O solvent for placement in OL^+ bond. Thus, considering equilibrium 2 as $PhXn^+ + 2L_2O = PhXnOL + L_3O^+$ and assuming H and D have the same opportunity for placement in L_2O and PhXnOL, the EIE of the equilibrium

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can be estimated to be $1/(0.69)^3 = 3.04$, which is not far from the value of 2.69 determined from this work. Note that our value was determined under reaction conditions (in 80% L_2O/20% AN, 60 °C) different from those used to obtain the 0.69 value (in pure L_2O, 25 °C), which probably contributes to the difference between the two EIE values (2.69 vs 3.04).

Kinetics.[17] When the hydride donor *i*-PrOH present in low concentrations (≤0.327 M in this work) was added into the aqueous equilibrium system of $PhXn^+$ and PhXnOH during kinetic experiments, the equilibrium formation of the alcohol adduct (PhXnOPr-*i*)[17] would not be expected to compete with the existing hydration equilibrium 2. Moreover, even if the PhXnOPr-*i* were formed, the acetal-like structure would be readily hydrolyzed to PhXnOH that was further converted back to its cationic precursor in acidic solution. While this is most likely the case for the reaction in aqueous solution, the equilibrium formation of the alcohol adduct in pure AN solution might be expected since *i*-PrOH is the only nucleophile present in the reaction solution. However, a UV−vis spectral experiment showed that under the kinetic conditions where [*i*-PrOH] ≤ 0.327M, the formation of the PhXnOPr-*i* was not observed. This differs markedly from our earlier observations of the significant side-equilibrium formation of the PhXnOPr-*i*[17] in the same hydride-transfer reaction in the parent *i*-PrOH containing AN (1:1 v/v). Therefore, for the hydride-transfer reaction in aqueous solution, the reaction most likely follows mechanism 3 that involves the formation of the hydration product (PhXnOH) in a rapid side-equilibrium with the carbocation. While in AN, the equilibrium concentration of PhXnOPr-*i* is insignificant, which results in the simplification that k^{obs} is equal to k^H.

$$\mathrm{PhXnOH}+\mathrm{H^+} \underset{+\mathrm{H_2O}}{\overset{-\mathrm{H_2O}}{\rightleftharpoons}} \mathrm{PhXn^+}+i\text{-PrOH} \xrightarrow{k^H} \mathrm{PhXnH} + \mathrm{acetone}+\mathrm{H^+} \quad (3)$$

Determinations of the pseudo-first-order rate constants (k^{pfo}) of the hydride-transfer reactions were carried out at 60 °C in 80% H_2O/20% AN with $[PhXn]_0 = 0.15$ mM and $[i\text{-PrOH}]_0 \le 0.327$M and in AN with $[PhXn]_0 = 1$ mM and $[i\text{-PrOH}]_0 \le 0.327$M. In the latter solvent, reaction aliquots taken at different times were diluted with AN, and the resultant solutions were analyzed by UV−vis spectrophotometry and kinetic scans were recorded (Figure 1). The k^{pfo} were computed from the absorbance versus time data for the consumption of $PhXn^+$ at 373 nm (ε = 29,800 M^{-1} cm^{-1} (AN)) and rate = $d[PhXnH]/dt = -d[PhXn^+]/dt$. Since $PhXn^+$ partitions between the hydride-transfer product and its water adduct (eq 3) in aqueous solution, the rate definition becomes the following: rate = $d[PhXnH]/dt = -d\{[PhXn^+]_{eq} + [PhXnOH]_{eq}\}/dt$. Therefore, simply following the decay of the UV absorbance of $[PhXn^+]_{eq}$ is inappropriate to evaluate the rate constant for the formation of PhXnH. Also since both PhXnH and PhXnOH absorb over the entire wavelength range, it is not possible to determine the k^{pfo} under these circumstances. Thus, it is necessary to take into account the expression $[PhXn^+]_T = [PhXn^+]_{eq} + [PhXnOH]_{eq}$ in order to evaluate the rate of formation of PhXnH. This was accomplished by diluting aliquots of the reaction mixtures with AN/water (3:1, v/v) containing 3 M $HClO_4$ to provide samples for the spectrophotometric determination of $[PhXn^+]_T$. The $HClO_4$ concentration is high enough to convert all of the PhXnOH

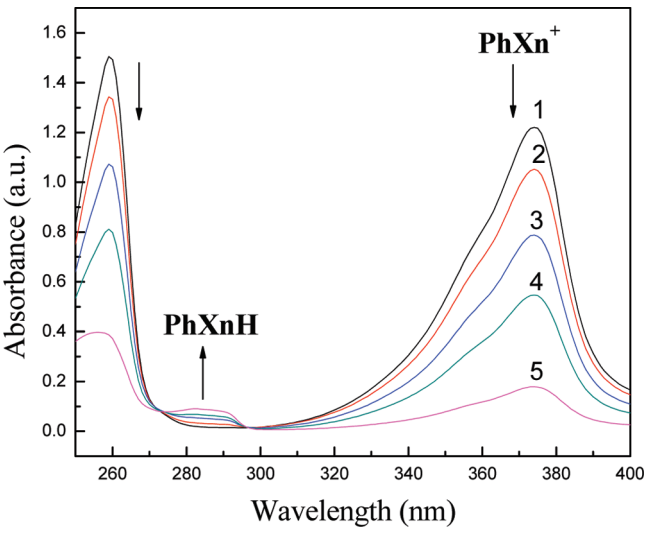

FIGURE 1. Kinetic scans determined by analysis of the reaction aliquots, taken from the reaction mixtures of $[PhXn^+]_0 = 0.001$ M and $[i\text{-PrOH}] = 0.0816$ M in AN at 60 °C. The reaction aliquots were diluted by 25 times with AN. Reaction times for spectra 1−5 are 30, 150, 359, 600, and 1295 min, respectively.

(maximum concentration of 4×10^{-5} M) in the diluted reaction aliquots to $PhXn^+$. Under these conditions, the rate of formation of PhXnH is equal to the rate of decrease of $[PhXn^+]_T$. The rate law of the reaction in aqueous solution derived from mechanism 3 is shown in eq 4.

$$\begin{aligned} \text{rate} &= d[\mathrm{PhXnH}]/dt = -d[\mathrm{PhXn^+}]_T/dt \\ &= k^H[\mathrm{PhXn^+}]_{eq}[i\text{-PrOH}] \\ &= k^H[\mathrm{PhXn^+}]_T[i\text{-PrOH}][\mathrm{H^+}]/([\mathrm{H^+}]+K) \\ &= k^{obs}[\mathrm{PhXn^+}]_T[i\text{-PrOH}] \end{aligned} \quad (4)$$

Effect of $[H^+]$ on k^{pfo}. The kinetics of the hydride-transfer reaction were not measurable in neutral aqueous solution due to low $[PhXn^+]_{eq}$. Sufficient acid was thus added to ensure that $PhXn^+$ was present in high enough concentrations for a measurable reaction to occur (see eq 5). Care had to be taken so that $[H^+]$ was not so high that the protonation of *i*-PrOH became significant, making the quantification of *i*-PrOH difficult. To this point, a pK_a value (−3.2) of the isopropyloxonium ion (*i*-$PrOH_2^+$) in water estimated by Bartlett[22] gave us some guides for selecting the $[H^+]$ range for study. According to rate law 4, k^{obs} should increase with increasing $[H^+]$ and reach saturation at $[H^+] \gg K$ when $[PhXn^+]_{eq} = [PhXn^+]_T$. Under the kinetic saturation conditions, k^{obs} should be equated to k^H.

The k^{pfo} ($= k^{obs}[i\text{-PrOH}]$) was determined for the reaction with $[i\text{-PrOH}]_0 = 0.327$ M in the presence of HBF_4 with concentrations ranging from 0.030 to 1.90 M that were not expected to favor the equilibrium formation of *i*-$PrOH_2^+$ (Figure 2). The results show that k^{pfo} increases with increasing $[H^+]$, tends to saturate when $[H^+]$ is increased to about 0.5−1.0 M, but unexpectedly decreases afterward. This kinetic behavior seems inconsistent with the rate law 4 or

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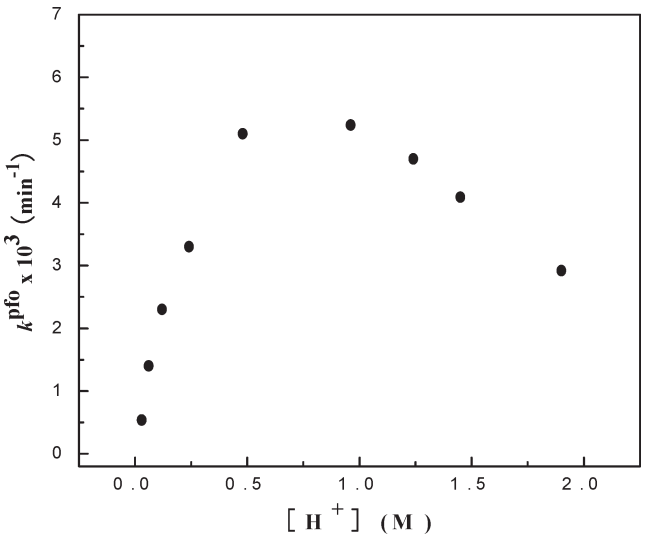

FIGURE 2. Observed pseudo-first-order rate constants (k^{pfo}) of the hydride-transfer reaction as a function of [H^+] in 80% H_2O/20% AN at 60 °C ([$PhXn^+$]$_0$ = 0.00015 M, [*i*-PrOH]$_0$ = 0.327 M). [H^+] are 0.03, 0.06, 0.12, 0.24, 0.48, 0.96, 1.24, 1.45, and 1.90 M, respectively. The corresponding k^{pfo} are 0.000536, 0.00141, 0.00230, 0.00332, 0.00510, 0.00524, 0.00470, 0.00409, and 0.00292 min^{-1}.

derived eq 5, which relates k^{pfo} to [H^+].

$$k^{pfo} = k^{H}[i\text{-PrOH}][H^+]/([H^+]+K) \qquad (5)$$

One may assume that the decomposition of AN by hydrolysis in acid to acetamide and further to acetic acid might be responsible for the decay of the reaction rate at higher [H^+] through changing the solvent composition. It has been shown, however, there was a very slow decomposition of AN in 50% water/50% AN containing high [$HClO_4$] (6 M) at room temperature; AN decayed by about 4% in 2 weeks.[23] Thus, AN decomposes very slowly under our reaction conditions, and this would not significantly affect the kinetic determinations of our reactions.

We consider the possibility that the observed dramatic rate reduction at high [H^+] may result from changes in ionic strength as the [HBF_4] increases. The latter brings about an increase in the polarity of the reaction medium, thereby affecting the rates of the reactions. This increase in polarity is expected to lower the rate of the reaction by destabilizing the hydride-transfer TS in which the overall 1+ charge on the reactant ($PhXn^+$) is dispersed between the two reactant moieties. To test this inference, we determined the effect of [H^+] on the observed value of k^{pfo} of the reaction under the conditions of constant ionic strength accomplished by adding an inert electrolyte ($NaClO_4$) to the acidic solutions, i.e., varying the [H^+] but keeping ([HBF_4] + [$NaClO_4$]) constant (1.24 M). As predicted, the kinetic saturation effect with depressed k^{pfo} at [H^+] ranging from 0.48 to 1.24 M was observed (Figure 3A), in accord with the "side-equilibrium" mechanism (eq 3). Results were then fit to a double reciprocal relationship ($1/k^{pfo} \approx 1/[H^+]$) obtained by transforming eq 5 (Figure 3B, R^2 = 0.990). From the obtained intercept and the slope of the latter linear plot, k^H and K were evaluated to be 0.0153 M^{-1} min^{-1} and 0.0339 M under the constant ionic strength conditions. Note that this latter K value is very different from the one determined under low [H^+] conditions in which about 50% of the cation was hydrated in the equilibrium solution (see the first paragraph of the Results section, K = 0.214 M). This suggests that the polar solvent with high salt concentration (or high ionic strength) facilitates the presence of the cation in the hydration equilibrium 2. Therefore, our observed bell-shaped relationship between k^{pfo} and [H^+] in Figure 2 resulted from the ionic strength changes of the kinetic solutions as [H^+] changed, i.e., the increase in ionic strength as increasing [H^+] lowers the rate of the hydride-transfer step and shifts the hydration equilibrium to the cation side.

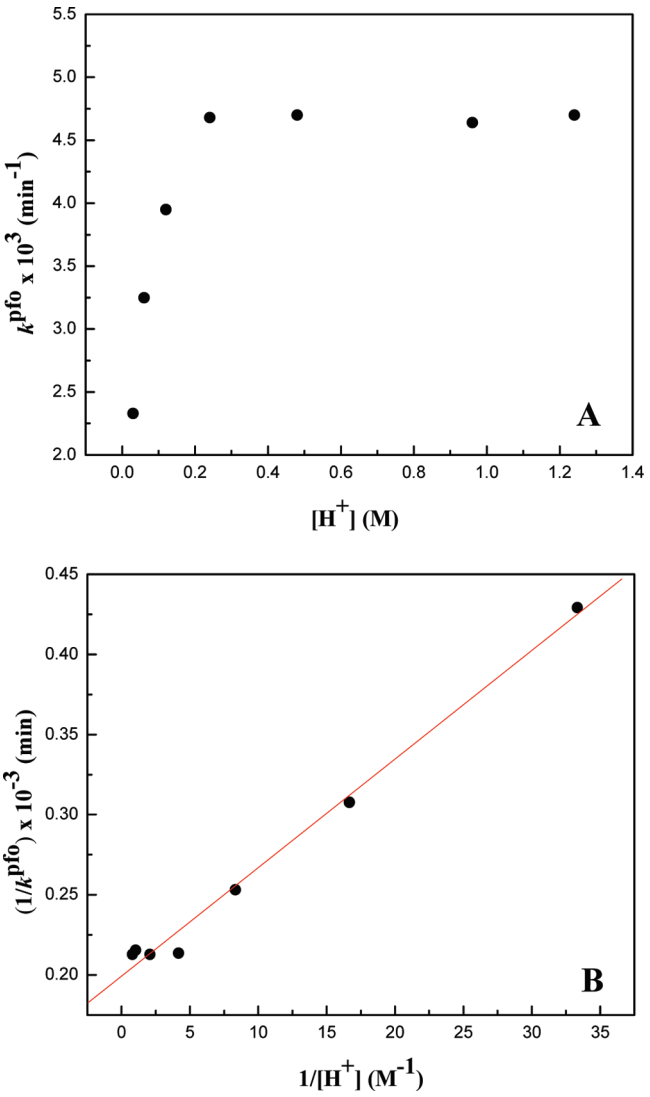

FIGURE 3. Observed pseudo-first-order rate constants (k^{pfo}) of the hydride-transfer reaction as a function of [H^+] (A) and the corresponding double reciprocal plot ($1/k^{pfo} \approx 1/[H^+]$) (B), in 80% H_2O/20% AN containing $NaClO_4$ at 60 °C ([$PhXn^+$]$_0$ = 0.00015M, [*i*-PrOH]$_0$ = 0.327 M, and [H^+] + [$NaClO_4$] = 1.24 M). [H^+] used are 0.03, 0.06, 0.12, 0.24, 0.48, 0.96, and 1.24 M, respectively. The corresponding k^{pfo} are 0.00233, 0.00325, 0.00395, 0.00468, 0.00470, 0.00464, and 0.00470 min^{-1}.

Inverse Kinetic Solvent Isotope Effect in Aqueous Solution. A comparison of the k^{pfo} for the reactions in H_2O and D_2O systems with the same alcohol concentrations (0.327 M) at low

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TABLE 1. Pseudo-First-Order Rate Constants (k^{pfo}) as a Function of [*i*-PrOH] in 80% H_2O/20% AN and in AN

in 80% water/20% AN at 52 °C[a,b]		in AN at 60 °C[b,c]	
[*i*-PrOH] (M)	$k^{pfo} \times 10^3$ (min^{-1})	[*i*-PrOH] (M)	$k^{pfo} \times 10^3$ (min^{-1})
0.654	5.49	0.327	5.67
0.327	2.75	0.164	2.70
0.164	1.37	0.0820	1.34
0.0820	0.673	0.0410	0.535

[a] $[PhXn^+]_0$ = 0.15 mM, $[H^+] = [HBF_4]$ = 0.96 M. [b] Based on three determinations. [c] $[PhXn^+]_0$ = 1 mM.

$[H^+]$ (0.06 M) gives the observed kinetic solvent isotope effect, $KIE_{D2O}{}^{pfo}$ (= $k^{pfo}(H_2O)/k^{pfo}(D_2O)$), which was evaluated to be equal to 0.53, i.e. an inverse solvent KIE was observed. Note that $KIE_{D_2O}{}^{pfo}$ is also equivalent to $KIE_{D_2O}{}^{obs}$, which is defined by the second-order-rate constant (k^{obs}).

The effect of acid concentration on the rate of the reaction in AN was also investigated. No apparent effect was observed, indicating that the formation of the alcohol adduct (PhXnOPr-*i*) is insignificant in AN.

Effect of [*i*-PrOH] on k^{pfo}. The effects of [*i*-PrOH] on the rates of the reactions in pure AN and in aqueous solution with $[H^+]$ = 0.96 M were determined (see Table 1). The results show that the reactions are first-order in the hydride donor (*i*-PrOH) in both solvents. This is consistent with the proposed mechanism 3. Second-order-rate constants (k^{obs}) can therefore be calculated by dividing the k^{pfo} by [*i*-PrOH].

Kinetic Isotope Effects at the Three H/D positions in *i*-PrOH. The effects of deuteration at the α- and β-C in *i*-PrOH on the kinetics of the reactions were determined by comparing the k^{pfo} determined in the presence of *i*-PrOH-2-d and $(CD_3)_2CHOH$ with that for the reaction of the normal *i*-PrOH under the same alcohol concentration conditions. Since deuterium substitution at either position has no effect on the hydration equilibrium, KIE^{pfo} is equal to the KIE of the net hydride-transfer process in both cases and are referred to as $KIE_{\alpha\text{-}D}{}^{H}$ and $KIE_{\beta\text{-}D6}{}^{H}$, respectively. Note that $KIE_{\beta\text{-}D6}{}^{H}$ is a cumulative effect of six β-C−D bonds. These results are listed in Table 2. The $KIE_{\alpha\text{-}D}{}^{H}$ in both solvent systems are primary (3.2 (AN, 60 °C) and 3.2 (H_2O, 54 °C)), while the $KIE_{\beta\text{-}D6}{}^{H}$ at 60 °C are secondary (1.05 (AN)) and 1.16 (H_2O)).

The KIE at the OH/OD positions in *i*-PrOH, referred to as KIE_{OD}, was calculated from the k^{pfo} of the reactions of *i*-PrOH and *i*-PrOD with same concentrations. In AN, $KIE_{OD}{}^{pfo}$ (= $KIE_{OD}{}^{obs}$) is equal to $KIE_{OD}{}^{H}$ (1.08 in Table 2). In aqueous solution, k^{pfo} of the reaction of *i*-PrOD must be determined in D_2O solvent in order to avoid H/D exchange. Therefore, $KIE_{OD}{}^{obs}$ in aqueous solution is given by $k^{pfo}(H_2O)/k^{pfo}(D_2O) = KIE_{D_2O}{}^{pfo} = KIE_{D_2O}{}^{obs}$. According to eq 4, $KIE_{OD}{}^{obs} = KIE_{OD}{}^{H}\{([H^+] + K(D_2O))/([H^+] + K(H_2O))\} = KIE_{D_2O}{}^{obs}$, so the $KIE_{D_2O}{}^{obs}$ encompasses the components of both the equilibrium isotope effect (EIE) and the $KIE_{OD}{}^{H}$. At $[H^+]$ = 0.06M, $KIE_{D_2O}{}^{obs}$ is equal to 0.53 (see previous sections), while the $KIE_{OD}{}^{H}$ under the same conditions was evaluated to be equal to 1.04 (Table 2), characteristic of a secondary KIE. Since the estimation of the KIE_{OD} in aqueous solution relies on the values of both *K* and $KIE_{D_2O}{}^{obs}$, the KIE value should contain the measurement errors of the two quantities. The uncertainty of this KIE value in aqueous solution would thus be higher than that in AN. Relevant KIEs obtained from the reactions in mixed solvent (*i*-PrOH/AN = 1/1) are also listed in Table 2 for comparison.[17] Note that, similarly, the larger uncertainty may be contained in the $KIE_{OD}{}^{H}$ estimated in this mixed solvent system, as it was separated from the observed KIE of the overall hydride-transfer reaction by excluding the EIE of the side-equilibrium formation of the PhXnOPr-*i* adduct, i.e., $KIE_{OD}{}^{H} = KIE_{OD}{}^{obs} \cdot EIE$.[17]

TABLE 2. Kinetic Isotope Effect of the Hydride-Transfer Step[a,b]

reaction solvent	$KIE_{\alpha\text{-}D}{}^{H}$	$KIE_{\beta\text{-}D6}{}^{H}$	$KIE_{OD}{}^{H}$
20% AN/80% H_2O[c]	3.2 ± 0.3 (2)[d]	1.16 ± 0.05 (4)	1.04 ± 0.02[e] (4)
AN[f]	3.2 ± 0.1 (4)	1.05 ± 0.03 (6)	1.08 ± 0.01 (4)
i-PrOH/AN (1:1 v/v)[f,g]	4.4	1.07	1.11

[a] At 60 °C, unless otherwise indicated; see texts for definitions of the KIEs. [b] Numbers in parentheses are times of determinations. [c] $[PhXn^+]_0$ = 0.15 mM, $[H^+]$ = 0.06 M, unless otherwise indicated. [d] At 54 °C, $[H^+]$ = 0.96 M. [e] Measurement error of the *K* was not considered; see text. [f] $[PhXn^+]_0$ = 1 mM. [g] Reference 17.

TABLE 3. Second-Order Rate Constants and the Corresponding Activation Parameters for the Net Hydride-Transfer Reaction in Aqueous[a] and AN Solutions

in 80% H_2O/20% AN (v/v)				in AN	
temp (°C)	$k^{obs} \times 10^{3b}$	*K* (M)[c]	$k^H \times 10^{3b}$	temp (°C)	$k^H \times 10^{3b}$
36	1.92	0.0895	2.10	36	3.48
43	3.73	0.0810	4.04	42	5.47
54	8.65	0.0746	9.32	54	9.88
60	15.9	0.0425	16.6	60	16.5
67	25.4	0.0450	26.6	67	24.8
$E_a{}^H$ = 71.6 ± 1.6 kJ/mol				$E_a{}^H$ = 58.3 ± 2.4 kJ/mol	
$\triangle H^{\ddagger H}$ = 68.9 ± 1.6 kJ/mol				$\triangle H^{\ddagger H}$ = 55.6 ± 2.4 kJ/mol	
$\triangle S^{\ddagger H}$ = −107.3 ± 4.4 J/mol K				$\triangle S^{\ddagger H}$ = −146.1 ± 7.9 J/mol K	

[a] Containing $[H^+]$ = 0.96 M. [b] M^{-1} min^{-1}. [c] Equilibrium constant of the equilibrium 2 in the aqueous solution containing $[H^+]$ = 0.96 M.

Kinetic Temperature Effects. The k^{pfo} for the reactions in both solvents with certain alcohol concentrations was also determined at various temperatures and was converted to the second-order-rate constants (k^{obs}). In AN, k^{obs} is equal to k^H (Table 3). In aqueous solution, k^{obs} were determined under the condition of $[H^+]$ = 0.96 M (Table 3). According to eq 5, a conversion from k^{obs} to k^H thus needs the *K* values determined under the specific acid conditions, i.e., specific ionic strength conditions. Table 3 thus also includes the *K* and derived k^H for the reactions in the aqueous solution as a function of temperature. Note that the *K* = 0.0425 M determined under $[H^+]$ = 0.96 M and 60 °C conditions (Table 3) may be comparable to the slightly smaller *K* = 0.0339 M derived from the aforementioned double reciprocal correlation between $1/k^{pfo}$ and $1/[H^+]$, which was obtained under the condition of larger "salt" concentrations, i.e., $([H^+] + [NaClO_4])$ = 1.24 M. Activation parameters including activation energies, activation enthalpies, and activation entropies for the hydride-transfer steps were evaluated using both the Arrhenius and the Eyring equations and are found in Table 3 as well. The activation parameters obtained in *i*-PrOH/AN in our previous work are $E_a{}^H$ = 71.1 ± 12.6 kJ/mol; $\triangle H^{\ddagger H}$ = 68.3 ± 12.6 J/mol; $\triangle S^{\ddagger H}$ = −106.9 ± 38.0 J/mol·K.[24] Within experimental

(24) For a direct comparison, the activation entropy of the reaction in *i*-PrOH/AN was calculated using the second-order rate constants ($M^{-1}s^{-1}$) converted from the pseudo-first-order rate constants reported in our previous work by dividing the alcohol concentration (6.54 M); see Table 5 in ref 17.

error, these values are close to those obtained in aqueous solution in this work.

Discussion and Conclusions

Solvent Effects on the Kinetics and Mechanism of the Hydride-Transfer Reactions. The hydride-transfer reaction, in aqueous solution or in mixed solvents (*i*-PrOH/AN), involves the formation of the water adduct (PhXnOH) or the alcohol adduct (PhXnOPr-*i*)[17] of the $PhXn^+$ cation via rapid equilibrium. While in pure AN solution, the formation of PhXnOPr-*i* is insignificant. These conclusions follow from the observation that acid does not affect the rate of the hydride-transfer reaction in AN but does increase the rates of the reactions in aqueous solution (Figures 2 and 3) and in mixed solvents of *i*-PrOH/AN (see our previous work[17]). The resulting hydride-transfer product, protonated acetone, exists transiently and undergoes rapid proton transfer to the basic species in the reaction solutions, forcing the reactions to go to completion.

We have reported an inverse kinetic solvent isotope effect (0.54) for this reaction in *i*-PrOH(D)/AN (1:1 v/v) and a larger than unity equilibrium isotope effect (EIE = K(*i*-PrOH)/K(*i*-PrOD)) (2.67) for the corresponding PhXnOPr-*i* formation equilibrium.[17] These results were consistent with the hydride-transfer mechanism involving the side-equilibrium formation of PhXnOPr-*i*. The EIE value shows that $[PhXn^+]_{eq}$ is higher in *i*-PrOD than in *i*-PrOH under the same reaction conditions, which is a consequence of the fact that the reaction is faster in *i*-PrOD than in *i*-PrOH. In this work, an inverse kinetic solvent isotope effect (0.53) was observed for the hydride-transfer reaction in water and a larger than unity EIE (2.69) for the hydration equilibrium of $PhXn^+$ was observed. Likewise, the latter EIE value indicates a higher $[PhXn^+]_{eq}$ in D_2O solvent than in H_2O, giving rise to an inverse solvent KIE. These results are consistent with mechanism 3 in which hydration is a side-equilibrium inhibiting the net hydride-transfer step. Our results also exclude an unlikely alternative mechanism in which the PhXnOH adduct is an intermediate that undergoes an acid-catalyzed S_N2-like substitution of OH with hydride acting as a nucleophile (Scheme 1). This mechanism is highly unlikely but is included for the sake of completeness.

We have suggested in our previous work that the TS of the hydride-transfer step in *i*-PrOH/AN (1:1 v/v) is the one in which a positive charge develops on the alcohol moiety during the reaction and is stabilized by H-bonding between the solvent O and the positively charged H of its α-C=$OH^{\delta+}$ ($\leftrightarrow$ α-$C^{\delta+}$−OH) moiety. This H-bonding interaction can be further supported by the comparison of the activation entropies of the reaction in aqueous solution with that in pure AN (−107.3 (aqueous) vs −146.1 J/mol·K (AN), Table 3). The observation that the activation entropy is 39 J/mol·K less negative for the reaction in aqueous solution than in AN suggests that the TS in the former solvent is much less ordered than in the latter solvent. This is consistent with the fact that water solvent loosens the associated TS structure by H-bonding interaction between water and the positively charged alcohol moiety in the TS. On the other hand, the observed large activation entropy in AN suggests that the hydride-transfer process would accompany a highly ordered and possibly cyclic TS structure that would be favored by the solvent that does not provide such H-bonding stabilization effect.

SCHEME 1. An Unlikely Alternative Mechanism in Aqueous Solution

$$PhXnOH + H^+ \rightleftharpoons PhXnOH_2^+ + i\text{-PrOH} \xrightarrow{-H_2O} PhXnH + \text{acetone} + H^+$$

On the other hand, a comparison of the activation energies of the reaction in aqueous solution (71.6 kJ/mol, Table 3) and in AN (58.3 kJ/mol) reveals that the reaction is about 13 kJ/mol less favored in the former than in the latter, despite the above suggested H-bonding stabilization effect on the TS of the reaction in the former solvent. This can be explained in terms of the H-bonding to the reactant alcohol O in the aqueous solution so that the ground-state reactant alcohol is less reactive toward donating hydride ion than in AN.

The activation parameters (E_a^H = 71.1 kJ/mol; $\triangle H^{\ddagger H}$ = 68.3 J/mol; $\triangle S^{\ddagger H}$ = −106.9 J/mol·K) that we obtained earlier in *i*-PrOH/AN mixed solvents are close to the ones observed in the aqueous solution within experimental error. We suggest that the parent alcohol in *i*-PrOH/AN would play a similar role as water does in deactivating the ground-state alcohol and stabilizing the TS.

Thus, AN medium would promote the reaction by activating the alcohol reactant through weak interaction with the electron pairs on alcohol O, whereas water and *i*-PrOH would facilitate the reaction by stronger H-bonding stabilization of the alcohol moiety of the TS. Interestingly, the results derived from this study of the non-enzymatic reactions are consistent with the traditionally accepted theories for enzymatic rate enhancement, i.e., the "ground-state destabilization theory" and the "TS stabilization theory". Furthermore, this H-bonding interaction in the TS is reminiscent to the substrate-enzyme interaction modes in alcohol dehyhrogenase reactions. For example, in a human lactate dehydrogenase reaction, H-bonding is known to be present between the hydroxyl H of the lactate substrate and the imidazole N of the His-193 during the reaction in the active site of the enzyme.[25]

KIE and the TS Structure. Kinetic isotope effects on the hydride-transfer step at the three H/D positions of *i*-PrOH can provide information about the structure of the TS. As expected, the KIE brought about by deuterium substitution at the α-C in all three reaction media are primary (Table 2, $KIE_{\alpha\text{-}D}^H$), indicating that the hydride-transfer step is rate-determining. The secondary KIEs, resulting from the six β-C−H/C−D bonds ($KIE_{\beta\text{-}D6}^H$) and the O−H/O−D bonds (KIE_{OD}^H) (Table 2), are consistent with a partially positively charged alcohol moiety in the TS. The former is a result of the orbital overlap between the β-C−H σ orbital and the p orbital on the carbon of the α-$C^{\delta+}$−OH in the TS, affecting the vibration of the β-C−H bonds. The latter results from the effect of the developing positive charge on the vibration of the O−H bond in the α-C=$OH^{\delta+}$ moiety of the TS. Since the β-C−H and the O−H bonds are more easily weaken and therefore more readily allow dispersion of the positive charge on the β-CH_3−$C^{\delta+}$−OH moiety as compared to the corresponding C−D/O−D bonds, normal secondary

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KIEs resulted. Note that the observed primary α-C−D KIE and secondary O−D KIE indicate that the deprotonation from the O−H bond during the overall alcohol oxidation to the carbonyl compound is not concerted with but after the rate-limiting hydride-transfer step.

$KIE_{\alpha\text{-}D}{}^{H}$ in *i*-PrOH/AN (4.4) is larger than those observed in the aqueous and AN solutions (3.2). The difference is an indication that the transferring H is more equally bound to reactive C sites of both reactants in the former than in the latter two solutions. Similar magnitudes of β-D KIEs in *i*-PrOH/AN (1.07) and in AN (1.05) suggest that the electron density of the α-C of the alcohol moiety of the TSs in both solutions would be the same. The slightly larger one observed in acidic aqueous solution (1.16) may be explained in terms of the H-bonding stabilization of the lone pairs of electrons on the hydroxyl O so that the developing positive charge in the TS is more localized at the α-C. Unlike the β-C−H(D) bond in the TS whose vibrations were not expected to be affected by solvation, the alcohol hydroxyl O−H(D) vibrations would be influenced through H(D)-bonding to the solvent electronegative atom. The O−D KIE magnitude would thus be affected by both the positive charge density on O and the strength of the solvation effect on the O−H(D) group. Since how solvation affects the O−H(D) vibrations is not known, a quantitative comparison between the O−D KIEs in three solvent systems may thus be meaningless (1.04 (aqueous), 1.08 (AN), 1.11 (i-PrOH/AN), Table 2).

Comparison to the β-D Isotope Effects in the Non-enzymatic and Enzymatic Reactions in Literature. While the O−D KIE has never been determined for either non-enzymatic or enzymatic hydride-transfer reactions involving alcohols in literature, a β-D KIE value of 1.19 was reported by Cleland and co-workers for the oxidation of lactate-β-d_3 mediated by lactate dehydrogenase.[26] To the knowledge of the authors, this is the only β-D KIE reported by other research groups for such hydride-transfer reactions. This value (1.06/β-D) is much larger than the corresponding ones observed in our non-enzymatic reactions (1.006/β-D in AN, 1.008/β-D in *i*-PrOH/AN, 1.02/β-D in aqueous solution, Table 2), suggesting that more positive charge is developed at the alcohol moiety of the TS in the enzymatic reaction than in non-enzymatic reactions. In addition to this experimental determination of the β-D KIE, theoretical calculations were carried out to evaluate the hyperconjugation stabilization effect by β-C−H(D) bonds on the adjacent hydroxy-carbocations (C^+−OH). For example, a calculated isotope effect value of 1.32 per three deuteriums on protonation of acetaldehyde was reported by Hess et al.,[27] whereas a much smaller value of 1.19 per six deuteriums on protonation of acetone was calculated by Alston et al.[28]

In conclusion, the rate constants of the hydride-transfer reactions from *i*-PrOH to $PhXn^+$ as well as the corresponding deuterium KIEs at the three H/D positions of *i*-PrOH and the activation parameters in pure AN and in aqueous solution (80% H_2O/20% AN) were determined. These results were compared with those observed in mixed solvents (50% *i*-PrOH/50% AN) that we reported earlier and the β-D KIE for a lactate dehydrogenase reaction found in literature. Protic solvents, water and alcohol, stabilize the hydride-transfer TS by H-bonding interaction between their hydroxyl O and the positively charged hydroxyl H of the alcohol moiety in the TS but retards the reaction by tying up the lone pairs of electrons on the OH of the ground-state reactant alcohols by solvation/H-bonding. Both trends were reversed in pure AN due to the lack of these H-bonding effects. Positive charge developing in the TS is delocalized over the alcohol α-C−OH group. Our results are important for the understanding of the TS of the alcohol dehydrogenase reactions, especially for those enzymes that do not contain a Zn(II) cofactor in the active sites, such as lactate dehydrogenases. In the alcohol dehydrogenases with a Zn(II) cofactor, the hydride source is most likely a zinc alkoxide. Our study suggests that in the former dehydrogenases, the alcohol group would be oxidized via hydride transfer from the α-C to the NAD^+ coenzyme followed by the rapid deprotonation from the hydroxyl O to the nearby basic species in the active sites of the enzymes, rather than via a concerted hydride−proton transfer process.

Experimental Section

General Procedures. 9-Phenylxanthylium tetrafluoroborate ($PhXn^+BF_4^-$) was synthesized according to the published procedure.[29] 2-Propanol-d_6 ($(CD_3)_2CHOH$) was prepared by the reduction of acetone-d_6 with $NaBH_4$ according to a procedure described in literature,[30] and the D content was determined to be 99.4% per C−D bond. Commercially available *i*-PrOD, *i*-PrOH-2-d, and isopropyl alcohol were all purified by distillation over dry K_2CO_3. Acetonitrile was distilled twice.

Determination of the Equilibrium Constant (*K*). The equilibrium constant (*K*) of the equilibrium between $PhXn^+$ and PhXnOH in aqueous solution (80% water/20% AN or with 80% D_2O) was determined spectrophotometrically (UV−vis) by recording the absorbance at 373 nm attributable to $PhXn^+$. The equilibrium was established by adding a certain amount of the PhXnOH stock solution in AN to the 80% water/20% AN solution of various concentrations of H^+ (= $[HBF_4]$) at 60 °C ($[PhXnOH]_0 = 2 \times 10^{-5}$ M). The $[H^+]$ range (about 0.04−0.16 M) was chosen so that about 30−60% of the PhXnOH were converted to the cation. Determinations based on three acid concentrations were performed, and the average $K(H_2O)$ and $K(D_2O)$ together with their standard deviations were reported. For the *K*s determined under high acid concentration ($[H^+] = 0.96$ M) and different temperature conditions, only the particular acid concentration was used. In the latter case, $[PhXn^+]_0 = 0.00015$ M, which was same as the initial concentration of the $PhXn^+$ in the kinetic solutions, was used, and the absorbance at 477 nm, which is also attributable to the cation, was recorded for the calculation of the $[PhXn^+]_{eq}$ and the *K* values.

General Kinetic Determination Procedure.[17] An 80 μL or 12 μL portion of a 0.1 M stock solution of $PhXn^+$ in AN was added to 8 mL AN or aqueous solution containing *i*-PrOH in a well sealed 10 mL reaction vial, which was thermostatted in a water bath at the desired temperature (< 70 °C). About 0.3 mL aliquots of the reaction were periodically taken into sample vials precooled in ice. The samples were immediately placed in a freezer (∼ −20 °C)

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until 6−8 reaction aliquots within 1−3 half-lives of the reaction had been collected. The aliquots of the reactions were then analyzed by dilution of 80 μL of them in 1.92 mL of AN/water (3:1, v/v) containing 3 M $HClO_4$ by UV−vis spectroscopy, an acid concentration that is high enough to convert all of the PhXnOH in equilibrium with the $PhXn^+$ in the aqueous aliquots to $PhXn^+$. For the reactions in AN, analyses using AN as a diluting agent gave the same results within experimental error. The corresponding UV spectra at different reaction times, i.e., the kinetic scans, were then obtained. Absorbance (Abs) decreasing with time (t) at 373 nm due to the total $PhXn^+$ absorption was recorded (also see Results). The obtained Abs−t data were fit to the first-order integrated rate equation, $-\ln(\text{Abs}) = k\cdot t + \text{constant}$, and the slope of the linear plot of $-\ln(\text{Abs})$ vs t was taken as the pseudo-first-order rate constant (k^{pfo}) of the reaction. The linear plots usually had regression coefficients (R^2) greater than 0.995. Each kinetic run was repeated at least 3 times in most cases. The KIEs were obtained by parallel determinations and taking the ratios of the pseudo-first-order rate constants observed for the reactions involving normal and deuterated *i*-PrOH, respectively.

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