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Isotopic Fractionation Factors of Intermolecular Hydrogen Bonds in Water

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Association constants for $N\text{-}H^+\cdots{}^-O$ hydrogen bond formation between substituted ammonium dications and phenolate ion were measured in water and deuterium oxide at 25°C and 2.0 M ionic strength. In combination with isotopic fractionation factors for phenol and the conjugate diacid of 1,2-ethanediamine determined by ^{13}C NMR spectroscopy, these yield isotopic fractionation factors for amine dication-phenolate ion hydrogen bonds in water: $\phi_{AB} = 0.69$ for 1,2-propanediamine dication with a pK difference between donor and acceptor, $\Delta pK_a = -0.45$, $\phi_{AB} = 0.88$ for 1,2-ethanediamine dication ($\Delta pK_a = -2.1$), and $\phi_{AB} = 1.1$ for piperizine dication ($\Delta pK_a = -3.5$). The hydrogen bond association constants follow Brønsted correlations $\alpha = 0.19$ in water and $\alpha = 0.27$ in deuterium oxide. The results are consistent with a double-minimum potential with a significant barrier for motion across the hydrogen bond. © 1992 Academic Press, Inc.

INTRODUCTION

Solvent isotope effects are among the most useful tools for analyzing the mechanistic features of enzyme-catalyzed reactions. Thus kinetic solvent isotope effects can give information about the structural, solvational, and hydrogen-bonding features at the active site for enzyme-bound ground states and transition states (*1–5*). However, interpretation of these effects is sometimes hampered by the lack of a detailed understanding of isotope effects due to individual sites in large macromolecules, including unpredictable medium effects and enzyme structural changes (*6–8*). Because of the well known importance of hydrogen bonding in enzyme structure and catalysis, we report here studies of solvent isotopes effects on simple intermolecular hydrogen bonds in water and their implications for the interpretation solvent isotope effects in enzyme reactions.

Equilibrium solvent isotope effects, ${}^{D_2O}K$, can be expressed in terms of contributions from n individual sites in the ground (reactant) state and m sites in the product state according to (*4, 9*)

$$ {}^{D_2O}K = \prod_i^n \phi_{RS}^i \Big/ \prod_j^m \phi_{PS}^j, \qquad [1] $$

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where the isotopic *fractionation factor* ϕ for a given solute AH which may be a stable species or a reaction intermediate (or transition state, in the case of kinetic solvent isotope effects) is defined for the hypothetical isotopic exchange equilibrium of Eq. [2].

$$\mathrm{AH} + \mathrm{DOH} \overset{\phi_{\mathrm{AH}}}{\rightleftharpoons} \mathrm{AD} + \mathrm{HOH} \qquad [2]$$

Qualitatively, ground state fractionation factor can be viewed as a measure of the "stiffness" of the A–H bond relative to that of bulk water. Solutes in more shallow potentials with smaller force constants than the O–H bonds of liquid water have fractionation factors $\phi < 1$. Thiols in particular have $\phi_{\mathrm{SH}} \approx 0.5$ due to the greater polarizability of sulfur compared to oxygen. Organic acids, amines, and alcohols typically have fractionation factors close to one. For transition states, a proton-in-flight usually gives $\phi_{\mathrm{TS}} = 0.1$ to 0.3 (*1, 4*).

There is extensive evidence for small fractionation factors $\phi_{\mathrm{AHA^-}} \approx 0.3$–$0.4$ for symmetrical O–H$\cdots{}^-$O hydrogen bonds in nonaqueous solvents (*10, 11*).[2] These have been interpreted in terms of strong, low-barrier hydrogen bonds where the lowest vibrational level is just above the central maximum of a one-dimensional double-minimum potential with the proton shared equally between the donor and acceptor oxygens as shown in Fig. 1. Still stronger hydrogen bonds will have a single-minimum potential and are expected to have slightly larger fractionation factors, while weak symmetrical hydrogen bonds can be described by a double-minimum potential with a significant central barrier for motion across the hydrogen bond with fractionation factors close to the parent acid.

Low-barrier hydrogen bonds may be particularly relevant to understanding and interpreting small fractionation factors observed in enzyme reactions, if the solvation properties of enzyme active sites are like dipolar aprotic solvents (*12*). For example, the low fractionation factors observed for binding of 2-deutero tartronate semialdehyde phosphate (TSP)[3] to enolase (*13*) or the binding of 8-oxoadenosine to adenosine deaminase (*14*) have been interpreted as evidence for low barrier hydrogen bonds along the reaction pathway of these enzymes (*12*).

In contrast to organic solvents, surprisingly little is known about fractionation factors of intermolecular hydrogen bonds in water (*10*). In large part this is because hydrogen bonding association constants K_{AB} in water (Eq. [3]) depend upon the difference in the stabilities of the hydrogen bonds on both sides of Eq. [3], and not the absolute strength of the A–H$\cdots$B bond, so that intermolecular hydrogen bonding in water is weak (*15, 16*).

$$\mathrm{AH}\cdots\mathrm{OH_2} + \mathrm{B}\cdots\mathrm{HOH} \overset{K_{\mathrm{AB}}}{\rightleftharpoons} \mathrm{AH}\cdots\mathrm{B} + 2\,\mathrm{H_2O} \qquad [3]$$

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[2] The term symmetrical used here refers to the difference between the donor and acceptor pK equal to zero and not necessarily to the location of the central proton.

[3] Abbreviations used: TSP, tartronate semialdehyde phosphate; NMR, nuclear magnetic resonance; EDA, 1,2-ethanediamine; DAP, 1,3-propanediamine; PIP, piperizine; D_2O, deuterium oxide; DMSO, dimethyl sulfoxide.

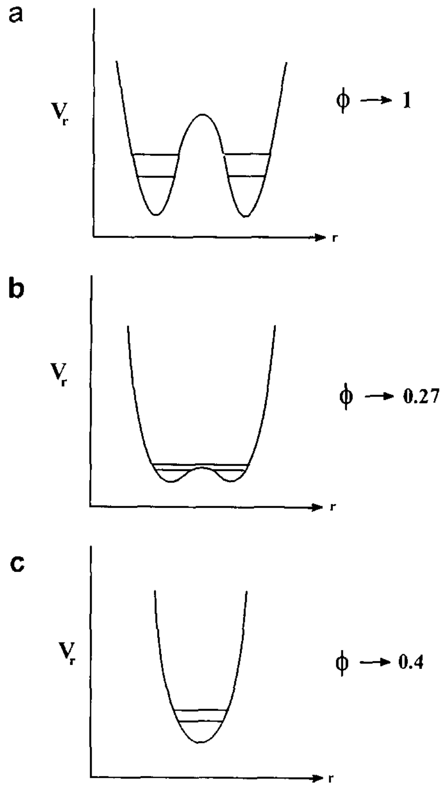

FIG. 1. Schematic diagram illustrating one-dimensional potential functions with zero point energy levels for H and D for (a) a double-minimum potential with a significant central barrier; (b) a double-minimum potential with a low central barrier; (c) a single-minimum potential; after Kreevoy and Liang (*11*). The fractionation factors are corrected for bending frequencies and represent qualitative trends.

It is frequently assumed that such hydrogen bonds will have $\phi_{AB} = 1$ (*1, 2, 5*). Hydrogen-bonded transition states (also termed solvation catalytic bridges) have been suggested to have $\phi_{TS} = 0.3$–0.6 in water (*2, 17–19*) but alternate interpretations involve heavy atom and protonic motion in the reaction coordinate of the transition state (*20*). Fractionation factors for *intramolecular* hydrogen bonds in water are only slightly less than 1 and may be strongly influenced by restricted rotation about the hydrogen bond. Typical values include $\phi_{AB} = 0.77$ for the O–H$\cdots{}^{-}$O hydrogen bond of maleate ion (*21*) and $\phi_{AB} = 0.90$ for the N–H$^{+}\cdots$H hydrogen bond of protonated 1,8-bis(dimethylamino)naphthalene (*22*).

We report here a systematic study of the fractionation factors of intermolecular hydrogen bonds between phenolate ion and substituted ammonium dications in water. The fractionation factors are close to 1 with the smallest fractionation factor $\phi_{AB} = 0.69$ corresponding to a fairly symmetrical hydrogen bond with a pK difference between donor and acceptor, $\Delta pK_a = -0.45$.

EXPERIMENTAL SECTION

Materials. Deuterium oxide (D_2O; 99.9 atom % D) was purchased from Isotec, Inc. Organic compounds were recrystallized or distilled before use. Deuterated ammonium chloride salts were obtained by isotopic exchange of the dihydrochloride salts in D_2O several times and were >90% deuterated.

Isotopic fractionation factors by ^{13}C NMR spectroscopy. Isotopic fractionation factors for phenol, 1,2-ethanediamine dihydrochloride (EDA Cl_2), and 1,2-ethanediamine were determined at 25°C and ionic strength 2 M using ^{13}C NMR spectroscopy as described by Jarret and Saunders (*21*). Spectra were acquired at 125.70 MHz on a Varian VXR 500S instrument using Wilmad 528-PP NMR tubes with WGS-5BL coaxial inserts. Interchange of the samples between the two compartments resulted in <0.11 Hz change in the frequency difference between H_2O and D_2O samples. A small correction for differences in the magnetic susceptibilities of H_2O and D_2O of 0.15 Hz for 50% H_2O/D_2O mixtures and 0.30 Hz for 100% D_2O samples was applied to the observed frequency differences according to Eq. [4] (*23*)

$$\Delta\delta_{\mathrm{corr}} = \Delta\delta_{\mathrm{obs}} + \frac{4}{3}\pi(\kappa_{\mathrm{R}} - x\kappa_{\mathrm{S}}) \times 10^6, \qquad [4]$$

where $\Delta\delta_{\mathrm{obs}}$ is the measured frequency difference in Hz between the reference sample in 100% H_2O and the sample containing D_2O, x is the mole fraction of deuterium in the solvent, and κ_{R} and κ_{S} are the diamagnetic susceptibilities of H_2O and D_2O, respectively, with $\kappa_{\mathrm{R}} = -0.7200 \times 10^{-6}$ for H_2O and $\kappa_{\mathrm{S}} = -0.6467 \times 10^{-6}$ for D_2O (*24*). The observed, statistically uncorrected, fractionation factor ϕ is given by

$$\phi = \frac{\Delta\delta_x(1 - x)}{(\Delta\delta_{1.0} - \Delta\delta_x)x}, \qquad [5]$$

where $\Delta\delta_x$ is the corrected frequency difference at a given mole fraction of deuterium and $\Delta\delta_{1.0}$ is the frequency difference corrected to complete deuteration of solute in 100% D_2O. The mole fraction of deuterium in the solvent was determined by a modification of the procedure of Kreevoy and Straub (*25*). The absorbance of liquid water at 975 nm was measured using a Hitachi U-2000 spectrophotometer. A calibration curve gave $r = 0.3985$ according to Eq. [5] of Ref. (*25*).

Spectrophotometic determination of association constants. Values of hydrogen bond association constants K_{AB} according to Scheme I were determined in water and deuterium oxide at ionic strength 2.0 M at 25°C as described by Stahl and Jencks (*15*). Spectra were recorded on a Hitachi U-2000 spectrophotometer. The apparent dissociation constants of phenol and substituted ammonium ion dications at 25°C and ionic strength 2.0 M were determined by potentiometric titration in water and deuterium oxide. Similar experiments in the two isotopic solvents were usually carried out on the same day. Solution pH and pD were measured with an Orion Model 701A pH meter and a Radiometer GK2321C combination electrode thermostated at 25°C. The value of pD was obtained by adding 0.40 to the observed pH of the solutions in D_2O (*26*). Isotopic dilution of the solvent by protium in the buffer or phenol

TABLE 1

Isotopic Fractionation Factors by ^{13}C NMR Spectroscopy[a]

Substrate	Concn. (M)	Ionic strength (M)	Observed frequency difference, $\Delta\delta$ (Hz)[b] 50% H_2O/D_2O	100% D_2O	ϕ_{AH}[c]
Phenol (C-α)	0.50	2.0	8.78	17.55	1.00
Phenol (C-o)	0.50	2.0	7.58	14.82	1.05
Phenol (C-α)[d]	0.50	0	5.42	10.36	1.13
Phenol (C-o)[d]	0.50	0	4.79	8.91	1.15
EDA · Cl_2	1.00	2.0	25.64	48.61	1.02
EDA	1.00	0	14.60	33.30	0.94

[a] At 25°C, unless otherwise indicated.

[b] All values determined at 125.79 MHz, unless otherwise indicated, with the signal in 100% H_2O as reference.

[c] Calculated according to Eq. [5] with corrections for differences in magnetic susceptibility of H_2O and D_2O, as described in the experimental section, and for the number of n statistically equivalent hydrogens in each solute according to $\phi_{AH} = \phi^{1/n}$; estimated uncertainty ±5%.

[d] At 67.90 MHz and 32°C (*21*).

never amounted to more than 2% in D_2O. Brønsted slopes were statistically corrected for the number of acid and base sites as described by Bell (*27*).

RESULTS

Fractionation factors for phenol and the conjugate diacid of 1,2-ethanediamine were determined at 25°C and ionic strength 2 M by measuring the isotopic shift of the ^{13}C NMR signals adjacent to the O–L or N–L bonds (L = H or D) as described in the experimental section. Results are summarized in Table 1.

Hydrogen bonding between phenol and ammonium ions in water can be described by the equilibria shown in Scheme I. The increase in the absorbance of the hydrogen-bonded $N\text{–}H^{+}\cdots{}^{-}O$ phenolate ion, with increasing concentration of amine dicationic buffers, was measured at constant pH values $< pK_a$ of phenol as described by Stahl and Jencks (*15*). These experiments yield values of the observed buffer association constants K_{buff} that depend upon the microscopic association constants of Scheme I and the fraction of diamine monocation free base, f_A, according to Eq. [6].[4]

$$K_{buff} = K_{AB} + (K_{BA} - K_{AB} - K_3)f_A \qquad [6]$$

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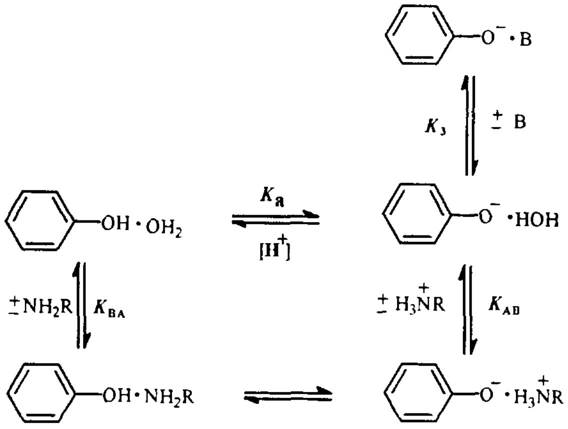
[4] The association constant K_3 in Eq. [6] and Scheme I refers to the weak hydrogen bonding of diamine monocations to phenolate ion. The derivation of Eq. [6] assumes that the extinction coefficients of the hydrogen-bonded phenolate ions are equal as discussed in detail in Ref. (*15*).



SCHEME 1

Extrapolation of values of K_{buff} to 100% dicationic buffer (f_A = 0) gives values for K_{AB}, the hydrogen bonding association constant for formation of the N–H$^+\cdots{}^-$O ion pair as shown in Fig. 2 for 1,2-ethanediamine dication in water and deuterium oxide. Table 2 summarizes apparent acid dissociation constants K_a^{AL} and hydrogen bond association constants K_{AB} for the conjugate diacids of 1,3-propanediamine, 1,2-ethanediamine, and piperizine.

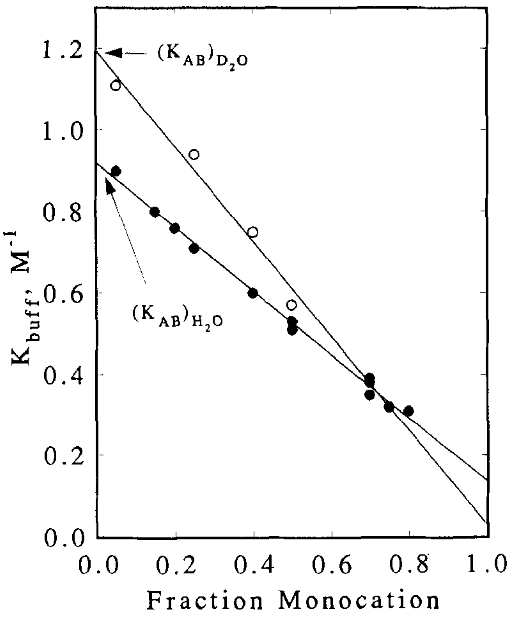


FIG. 2. Dependence of the observed association constant, K_{buff}, on the fraction of 1,2-ethanediamine dication in water (●) and deuterium oxide (○) at 25°C, ionic strength 2 M (KCl).

TABLE 2

Association Constants K_{AB} and Isotopic Fractionation Factors of $N{-}H^{+}\cdots{}^{-}O$ Hydrogen Bonds in Water and Deuterium Oxide[a]

	pK_a^{AL} [b]		K_{AB} (M^{-1})		
Amine conjugate diacid	H_2O	D_2O	H_2O	D_2O	ϕ_{AB}[c]
1,3-Propanediamine	9.30	9.66	0.45 ± 0.01	0.45 ± 0.01	0.69
1,2-Ethanediamine	7.65	8.18	0.92 ± 0.01	1.19 ± 0.03	0.88
Piperizine	6.27	6.76	1.10 ± 0.04	1.8 ± 0.20	1.1

[a] At 25°C, ionic strength 2 M (KCl).

[b] Apparent pK_{a1} measured at 2 M ionic strength by potentiometric titration.

[c] Calculated from Eq. [7]; see text.

DISCUSSION

The fractionation factors summarized in Table 1 are consistent with the known hydrogen bonding properties of these solutes in water. Thus the O–H and N–H bonds of phenol and 1,2-ethanediamine dication have fractionation factors close to 1 and are similar to fractionation factors $\phi_{OH} = 1.13$ of phenol (*21*) and $\phi_{NH_4} = 1.05$ of ammonium ion (*4, 28*), determined at low ionic strength. The fractionation factor of 1,2-ethanediamine is slightly smaller than the average fractionation factor of primary amines $\phi_{RNH_2} = 1.06$ determined by Jarret and Saunders at 35°C (*21*) and may reflect a weak intramolecular $N{-}H\cdots N$ hydrogen bond between the two nitrogens.

Values of $K_{AB} = 0.45\ M^{-1}$, $0.81\ M^{-1}$, and $0.91\ M^{-1}$ for the conjugate diacids of 1,3-propanediamine, 1,2-ethanediamine, and piperizine have been reported previously (*15*). Considering the uncertainties inherent in measuring these small association constants these values show good agreement with the values reported in Table 2. The hydrogen bond association constants follow statistically corrected Brønsted correlations $\alpha = 0.19$ in water and $\alpha = 0.27$ in deuterium oxide as shown in Fig. 3. The nonzero slopes show that the association constants do not simply represent formation of an electrostatic complex. The different slopes in the two isotopic solvents requires that the fractionation factors of intermolecular hydrogen bonds depend strongly on the difference in pK between the donor and acceptor groups, with the smallest fractionation factor for the most symmetric hydrogen bond.

The isotopic fractionation factor for the hydrogen-bonded ion pair, ϕ_{AB}, can be calculated for the equilibrium of Eq. [3] using a rearranged form of Eq. [1],[5]

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[5] Equation [7] assumes that (1) the fractionation factor for ammonium dications is independent of ammonium ion pK, (2) there is no isotopic fractionation in the 5 remaining $N^{+}{-}H$ bonds of 1,2-ethanediamine dication upon formation of the hydrogen-bonded complex, and (3) medium or transfer effects are neglected. The first two assumptions are reasonable in that hydrogen bonding of ammonium ions to water is known to be very weak (*29*). Transfer effects are difficult to evaluate experimentally (*30*), but the similar values for the free energies of transfer of KCl and NH_4Cl between H_2O and D_2O (*31*) are consistent with the absence of significant cationic medium effects.

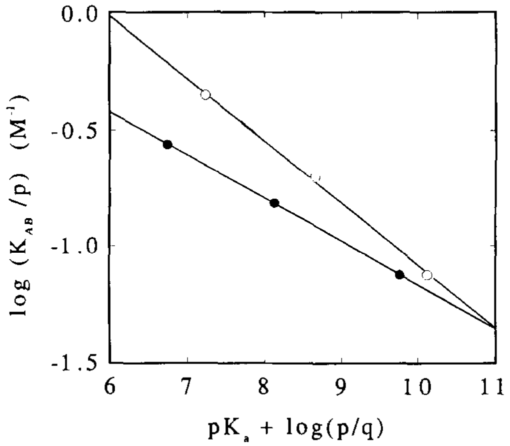

FIG. 3. Brønsted plots for hydrogen-bond association constants K_{AB} in water (●; α = 0.19) and deuterium oxide (○; α = 0.27) at 25°C, ionic strength 2 M (KCl). Statistical corrections for the number of acid sites (p = 6 for DAP and EDA; p = 4 for PIP) and base sites (q = 2) were made according to Bell (*27*).

$$\phi_{AB} = \phi_{AH}\,\phi_{B^-}/{}^{D_2O}K_{AB}, \qquad [7]$$

where ϕ_{AH} and ϕ_{B^-} are the fractionation factors for the ammonium dication and the phenolate ion, respectively, and ${}^{D_2O}K_{AB}$ is the solvent isotope effect on K_{AB}.[6] The fractionation factor of phenolate ion, ϕ_{B^-}, can be calculated from the solvent isotope effect on the apparent acid dissociation constant of phenol according to $\phi_{B^-} = \phi_{BH}/({}^{D_2O}K_a l^3)$, where ${}^{D_2O}K_a$ is the solvent isotope effect on the acid dissociation constant of phenol and l = 0.69 is the fractionation factor for hydronium ion. For phenol the apparent acid dissociation pK_a = 9.75 ± 0.005 and ${}^{D_2O}K_a$ = 4.69 ± 0.08 were determined potentiometrically at ionic strength 2 M. These values show reasonable agreement with pK_a = 9.85 at 2 M ionic strength (*15*) and pK_a = 10.0 with ${}^{D_2O}K_a$ = 4.17 at 0.01 M ionic strength (*34*) determined spectrophotometrically. These values along with ϕ_{BH} = 1.03 ± 0.05 (Table 1) give ϕ_{B^-} = 1.03/(4.69 × 0.69^3) = 0.67 ± 0.03. Table 2 summarizes values of ϕ_{AB}, calculated according to Eq. [7].

The fractionation factor ϕ_{AB} = 0.69 of 1,2-propanediamine dication-phenolate ion hydrogen bond corresponds to the most symmetrical hydrogen bond (ΔpK_a = −0.45) and is similar to fractionation factors for other symmetrical hydrogen bonds in water. A fractionation factor ϕ = 0.68 for the O–H$\cdots{}^-$O hydrogen bond of bis(formate)ion in concentrated aqueous salt solution has

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[6] The fractionation factor ϕ_{B^-} is sometimes referred to as a transfer or medium effect (*4*) but, as pointed out by Bell (*27*), this distinction can be artificial at times. The magnitude of ϕ_{B^-} is reasonable being intermediate between values of ϕ_{OH^-} = 0.43 for hydroxide ion (*22*) and ϕ_{RCOO^-} = 0.89 for acetate ion (*32*). The larger fractionation factors of less basic oxyanions are consistent with a decrease in solvation energy that reflects a decrease in hydrogen bond donation from the solvent to the lone pairs of less basic oxyanions (*33*).

been determined by ^1H and ^2H NMR spectroscopy (*35*) and $\phi = 0.6$ for the F–H$\cdots{}^-$F hydrogen bond of bis(hydrogen fluoride) ion in water (*4, 11*) that is not significantly different than the fractionation factor for the parent acid $\phi_{HF} = 0.61$ (*4*).

Most of the available data on intermolecular hydrogen bonding in water suggest that ionic hydrogen bonds can be represented by double-minimum potentials with a significant barrier for motion of the hydrogen across the barrier (Fig. 1a). It has been suggested that water interacts electrostatically with the electronegative groups of the hydrogen bond. This interaction lengthens the donor acceptor distance across the hydrogen bond increasing the central barrier and raising the fractionation factor relative to less polar solvents (*11*).[7] The small shift in λ_{max} of the phenolate ion toward the absorption maximum of phenol as the concentration of EDA Cl_2 is increased in water is consistent with a double-minimum potential even for fairly unsymmetrical N-H$^+\cdots{}^-$O hydrogen bonds in water (*15*). The negative difference in the ^1H and ^2H chemical shift, $\Delta[\delta(^1H) - \delta(^2H)]$, for bis-hydrogen fluoride ion in acetonitrile and hydrogen phthalate ion in CH_2Cl_2 provide evidence for low-barrier hydrogen bonds in these solvents (*10*). In water however, the fractionation factors are not significantly different than the parent acids ($\phi_{OHO} = 0.95$ for the hydrogen phthalate (*21*)), and the ^{18}O isotope effect on the ^{13}C chemical shift of hydrogen phthalate suggests that intramolecular O–H$\cdots{}^-$O hydrogen bonds in water are of the double-minimum type (*38*). Overall, the present results lend qualitative support to the conclusion that strong, low-barrier hydrogen bonds with low-fractionation factors require a relatively non-polar environment and fairly similar proton donor and acceptor p*K*s.

Implications for enzyme solvent isotope effects. Proteins are partially stabilized by N–H$^+\cdots{}^-$O salt bridges between Lys/His/Arg residues and Asp/Glu residues at physiological pHs. Since most salt bridges in proteins are highly solvent accessible (*39*), the fractionation factors close to 1 for the asymmetric N–H$^+\cdots{}^-$O hydrogen bonds with $\Delta pK_a = -2$ to -4 reported here suggest that the solvent isotope effects on protein conformations that are stabilized by salt bridges are likely to be small. This confirms the more general assumption that solvent isotope effects on protein stabilities are small (*1, 5*).[8]

In discussions of low-barrier hydrogen bonds in enzyme catalyzed reactions, it is important to note the assumption that the solvational properties of enzyme active sites are similar to the properties of dipolar, aprotic solvents like acetonitrile and DMSO. If this assumption is correct, the hydrogen-bonding interactions of small molecules in aqueous solution may not be relevant to similar interactions in the low effective dielectric of protein interiors.

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[7] However, the fractionation factor for hydronium ion $l = 0.79$ in acetonitrile suggests that organic solvents may interact strongly with charged hydrogen bonds, weakening the force constant for the O–H bond (*36*). In addition the simple two-dimensional picture in Fig. 1 may not be adequate to fully describe hydrogen bonding in polyatomic systems or condensed phases (*35, 37*).

[8] A potential caveat to this conclusion is suggested by the ab initio studies of Scheiner and Hillenbrand (*40*) who have suggested that small changes in hydrogen bond geometry may result in shifts in the relative p*K*s of donor and acceptor groups in proteins.

There is some theoretical (*41, 42*) and experimental (*43, 44*) evidence against the description of enzyme active sites as cavities of low dielectric, however. The similar pK_a values of amino acid side chains in proteins compared the intrinsic pK_a of the corresponding amino acid side chains in bulk water provides indirect evidence against a low active site dielectric (*41, 42*). Fersht and co-workers (*43*) have estimated apparent effective dielectrics (ε_{eff}) ranging from 50 to 100 for subtilisin on the basis of changes in the pK_a of the active site histidine with changes in charged surface residues by site-directed mutagenesis. These values are comparable to the dielectric constant of liquid water ε_{H_2O} = 78 at 25°C. Even in the specific case of enolase, where isotopic and X-ray structural measurements provide evidence for low-barrier hydrogen bonds (*13*), it appears that the active site is surrounded by a cluster of carboxylate and ammonium ion residues, as well as several water molecules. The oxygen of the putative water molecule that gives rise to the low isotopic fractionation factor is hydrogen-bonded to a histidine residue in the ground state binary complex. The low fractionation factor observed in this enzyme reaction suggests that the ion–dipole and dipole–dipole interactions that lengthen hydrogen bonds in water do not raise the fractionation factor of hydrogen bonds in the interior of proteins. Further theoretical work is needed in this area. The available data suggest that mechanistic assignments of low-barrier hydrogen bonds in enzyme reactions may be more reliable if they are based on both small fractionation factors and the observation of hydrogen-bonded complexes by X-ray crystallography rather than assignments based on small fractionation factors alone.

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