The change in hydrogen bond strength accompanying charge rearrangement: Implications for enzymatic catalysis

(ötense bonding/active site environments)

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ABSTRACT The equilibrium for formation of the intramolecular hydrogen bond (KHB) in a series of substituted salicylate monoanions was investigated as a function of ΔpK, the difference between the pK values of the hydrogen bond donor and acceptor, in both water and dimethyl sulfoxide. The dependence of log KHB upon ΔpK is linear in both solvents, but is steeper in dimethyl sulfoxide (slope ≈ 0.73) than in water (slope ≈ 0.05). Thus, hydrogen bond strength can undergo substantially larger increases in nonaqueous media than aqueous solutions as the charge density on the donor or acceptor atom increases. These results support a general mechanism for enzymatic catalysis, in which hydrogen bonding to a substrate is strengthened as charge rearrangements in going from the ground state to the transition state; the strengthening of the hydrogen bond would be greater in a nonaqueous enzymatic active site than in water, thus providing a rate enhancement for an enzymatic reaction relative to the solution reaction. We suggest that binding energy of an enzyme is used to fix the substrate in the low-dielectric active site, where the strengthening of the hydrogen bond in the course of a reaction is increased.

Electronic rearrangement typically occurs in the course of a reaction, resulting in changes in the charge density of functional groups on the reactant. This is shown in Scheme I for the example of the triosephosphate isomerase (TIM) reaction.

As the reaction proceeds from the ground state to the transition state, negative charge accumulates on the carbonyl oxygen, as reflected by the ~10 unit increase in the pK of this group. The pK of the carbonyl oxygen in the ground state is estimated to be ~2 to 3; the pK of the enol hydroxyl in a fully enolic transition state is estimated to be ~10 (1). The hydrogen bond (H bond) from a histidine residue of TIM to the carbonyl oxygen can be strengthened by this charge buildup (depicted by the darker dots in Scheme I). However, to obtain a rate enhancement relative to the solution reaction, the strengthening of an H bond to an enzymatic group in the course of a reaction must be greater than the strengthening of the corresponding H bond to water.

Could the environment of the enzyme active site increase the change in H bond strength accompanying charge rearrangements relative to that in water? To address this question, we used the aprotic organic solvent dimethyl sulfoxide (DMSO) as a crude mimic of the active site environment and investigated the energetics of the intramolecular H bond in a series of substituted salicylate monoanions in both DMSO and water. There is a larger increase in H bond strength in DMSO than in water as the pK values of the H bonding groups are varied. We suggest that a substantial amount of catalysis can be obtained by enzymes from the greater strengthening of H bonds accompanying charge rearrangements in nonaqueous environments than in aqueous solutions.

MATERIALS AND METHODS

Materials. The indicators 2,6-di-t-butyl-4-nitrophenol and 9-carboxymethylfluorene were synthesized from 2,6-di-t-butylphenol (Fluka) and 9-fluorenecarboxylic acid (Aldrich), respectively, as described (2–4). The indicator 2,4-dinitrophenol was from Sigma. Compounds 1–11 were from Aldrich. Compound 12 was a hydrolysis product of phthalide (Aldrich). Phosphoric acid, citric acid, and DMSO (~99.9%) were from J. T. Baker. Deuterated DMSO and water were from Cambridge Isotope Laboratories (Cambridge, MA). All compounds were of the highest commercial grade available and were dried in vacuo over P2O5 for >24 hr prior to use. Compounds with purity <98% were further purified by recrystallization.

Determination of pKHB in DMSO. Observed pK values (pKobs) of the carboxylic acid groups in compounds 1–11 in DMSO were measured by the overlapping indicator method at 25°C (2–4). The indicators used were 2,4-dinitrophenol, 2,6-di-t-butyl-4-nitrophenol, and 9-carboxymethylfluorene [pK values of 5.12, 7.30, and 10.35 in DMSO, respectively (2, 3)]. Each pK value in Table 1 was measured with two indicators whose pK values bracket that of the compound of interest. Standard deviations in the pKobs values from independent determinations varied from ±0.02 to ±0.14, with an average standard deviation of ±0.06.

Determination of pKobs in Water. Observed pK values in water were determined by spectral titrations. The spectra of 50 μM solutions of substituted salicylic acids were measured in buffered solutions with pH values from 0 to 6.0 at 25°C. The change in absorbance at appropriate wavelengths as a function of pH was fit to a titration curve. Absorbance changes were typically 0.2 to 0.3, and correlation coefficients of nonlinear least squares fits were >0.99. The buffers used were as follows: 1.0 M and 0.10 M hydrochloric acid (pH 0 and 1.0, respectively), 5 mM sodium phosphate (pH 1.6–3.0), and 5 mM sodium citrate (pH 2.8–6.0). The ionic strength was not held constant. The pK values determined from absorbance changes at two wavelengths differ by 0.02 units on average, and are within 0.2 unit of reported literature values (5). These small differences do not change the conclusion that log KHB has a small dependence on ΔpK in water. However, use of the same set of buffers for the pK determination of all compounds results in less scatter in the plots of log KHB (water) versus ΔpK and log KHB (DMSO) versus log KHB (water), presumably because of an increase in the precision of the data.

Abbreviations: DMSO, dimethyl sulfoxide; TIM, triosephosphate isomerase; SA, salicylic acid; LBH, low-barrier H bonds.
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Table 1. Formation constants and $\Delta \mathrm{pK}_a$ values of the H bond in substituted SA monomers in DMSO and water

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\mathrm{pK}_a^\text{obsd}$</th>
<th>$\mathrm{pK}_a^\text{int}$</th>
<th>$\Delta \mathrm{pK}_a^*$</th>
<th>log $K_{\text{HB}}^\text{H}$</th>
<th>log $K_{\text{HB}}^\text{H,O}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-OH-SA (1)</td>
<td>7.1</td>
<td>3.2</td>
<td>13.7</td>
<td>5.0</td>
<td>0.1</td>
</tr>
<tr>
<td>4-Br-SA (2)</td>
<td>5.5</td>
<td>2.7</td>
<td>11.9</td>
<td>4.4</td>
<td>0.8</td>
</tr>
<tr>
<td>4-OMe-SA (3)</td>
<td>7.1</td>
<td>3.2</td>
<td>13.1</td>
<td>4.9</td>
<td>0.9</td>
</tr>
<tr>
<td>5-Br-SA (4)</td>
<td>5.3</td>
<td>2.5</td>
<td>11.5</td>
<td>4.2</td>
<td>1.7</td>
</tr>
<tr>
<td>5-Cl-SA (5)</td>
<td>5.4</td>
<td>2.5</td>
<td>11.5</td>
<td>4.2</td>
<td>2.0</td>
</tr>
<tr>
<td>4-Me-SA (6)</td>
<td>7.0</td>
<td>3.0</td>
<td>12.7</td>
<td>4.7</td>
<td>2.2</td>
</tr>
<tr>
<td>SA (7)</td>
<td>6.6</td>
<td>2.9</td>
<td>12.4</td>
<td>4.6</td>
<td>2.2</td>
</tr>
<tr>
<td>5-F-SA (8)</td>
<td>5.8</td>
<td>2.5</td>
<td>11.6</td>
<td>4.2</td>
<td>2.7</td>
</tr>
<tr>
<td>5-Me-SA (9)</td>
<td>6.8</td>
<td>3.0</td>
<td>12.5</td>
<td>4.6</td>
<td>2.9</td>
</tr>
<tr>
<td>5-OMe-SA (10)</td>
<td>6.6</td>
<td>2.8</td>
<td>12.1</td>
<td>4.5</td>
<td>3.1</td>
</tr>
<tr>
<td>5-OH-SA (11)</td>
<td>7.1</td>
<td>2.8</td>
<td>12.1</td>
<td>4.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Only one significant number is reported for the $\mathrm{pK}_a$ values for clarity, though the more precise $\mathrm{pK}_a$ values were used to calculate log $K_{\text{HB}}$ and $\Delta \mathrm{pK}_a$.

$^*$Determined as described in Materials and Methods.

Values listed are the intrinsic $\mathrm{pK}_a$ values of the COOH groups, determined as described in Materials and Methods. The intrinsic $\mathrm{pK}_a$ values of the OH groups are not listed for simplicity, though they were used to calculate the $\Delta \mathrm{pK}_a$ values. These values can be obtained from $\mathrm{pK}_a^\text{int} = \mathrm{pK}_{\text{HB}}^\text{H} + \Delta \mathrm{pK}_a$ (see Materials and Methods).

$^\dagger$Obtained from the relationship: log $K_{\text{HB}} = \log(K_\text{HB}^\text{H}/K_\text{HB}^\text{H,O}) = \mathrm{pK}_a^\text{int} - \mathrm{pK}_a^\text{obsd}$, derived from Scheme II.

Determination of $\mathrm{pK}_a^\text{int}$ and $\Delta \mathrm{pK}_a$. The “intrinsic” $\mathrm{pK}_a$ ($\mathrm{pK}_a^\text{int}$, see also Results) represents the $\mathrm{pK}_a$ of the functional group (OH or COOH) expected from the polar and resonance effects of the substituents in the absence of H bonding (Scheme III).

![Diagram](https://via.placeholder.com/150)

$\Delta \mathrm{pK}_a$ represents the difference in the intrinsic $\mathrm{pK}_a$ values of the H bond donor and acceptor. Thus, the $\mathrm{pK}_a^\text{int}$ values of both the COOH and OH groups were determined to obtain the $\Delta \mathrm{pK}_a$ value for the H bond in each compound.

The $\mathrm{pK}_a^\text{int}$ value was estimated as the $\mathrm{pK}_a$ of the functional group in a corresponding compound with the OH group $\text{para}$ to the COOH instead of ortho, so that no intramolecular H bond can form (Scheme III). For salicylic acid (SA), the intrinsic $\mathrm{pK}_a$ of the COOH group was estimated as the $\mathrm{pK}_a$ value of 4-hydroxybenzoic acid [12.4 (6)]. 

For SA derivatives with a substituent X at the 4- or 5-position (X-SA), the intrinsic $\mathrm{pK}_a$ of the COOH group was calculated from:

$$\mathrm{pK}_a^\text{COOH}^\text{int} (X-SA) = \mathrm{pK}_a^\text{COOH}^\text{int} (SA) - \alpha_X \cdot \rho_{\text{COOH}}$$

where $\alpha_X$ is the Hammett constant describing the electron-accepting ability of the substituent X and $\rho_{\text{COOH}}$ is the slope of the linear dependence of $\mathrm{pK}_a^\text{COOH}$ on the $\sigma_X$ value of the substituent for benzoic acids. The intrinsic $\mathrm{pK}_a$ of the OH group of SA was obtained from the relationship:

$$\mathrm{pK}_a^\text{OH}^\text{int} (SA) = \mathrm{pK}_a^\text{OH} - \rho_{\text{COOH}} \cdot \rho_{\text{OH}}$$

where $\rho_{\text{COOH}}$ is the Hammett constant of a p-COOH substituent and $\rho_{\text{OH}}$ is the slope of the linear dependence of $\mathrm{pK}_a$ on the $\sigma$ value of the substituent for phenols. The intrinsic $\mathrm{pK}_a$ of the OH group in substituted salicylates was calculated from:

$$\mathrm{pK}_a^\text{OH}^\text{int} (X-SA) = [\mathrm{pK}_a^\text{OH}^\text{int} (SA) - \alpha_X \cdot \rho_{\text{OH}}]$$

The Hammett constants $\alpha_X$ and $\sigma_X$ were used for the ionization of COOH groups, since these constants are based on the $\mathrm{pK}_a$ values of benzoic acids (7, 8).

To account for the greater resonance contributions of substituents in the ionization of phenols than benzoic acids, the $\sigma_X$ scale was used for the $\mathrm{pK}_a$ values of the OH groups (7, 8). The effect of a p-COOH substituent on the $\mathrm{pK}_a$ of the phenol hydroxyl (i.e., $\sigma_X$) cannot be obtained because the COOH group deprotonates before the OH group does; the $\sigma$ value of p-COOH was used instead to estimate the intrinsic effect of the p-COOH group. Any error introduced by this approximation is constant for the entire series of compounds and therefore does not affect the slopes of plots of log $K_{\text{HB}}$ versus $\Delta \mathrm{pK}_a$ or the conclusions derived herein (see also below). The $\rho$ values used were 2.4 in DMSO and 1.0 in water for deprotonation of benzoic acids, and 5.3 in DMSO and 2.26 in water for deprotonation of phenols (4, 8).

The following suggest that the decrease of the $\mathrm{pK}_a$ values of carboxylic acids in SAs arise from the H bonding interactions in their monomers in both solvents, and that the determination of $\mathrm{pK}_a^\text{int}$ values described above are appropriate for the analysis herein. Ortho- and para-substituents generally have similar resonance and polar effects on the acidity of benzoic acids (7–9), so that the decreased $\mathrm{pK}_a$ values for SAs relative to the corresponding para-compounds can be attributed to H bonding. In addition, comparison of the $\mathrm{pK}_a$ values of benzoic acids with the same substituent at the 2- or 4-position suggests that ortho-specific effects from steric or electrostatic features of the OH and COOH groups are small, decreasing the $\mathrm{pK}_a$ of benzoic acids by < 1 pK unit in both solvents (see ref. 6 for a summary of the relevant $\mathrm{pK}_a$ values in DMSO; the corresponding aqueous $\mathrm{pK}_a$ values can be found in ref. 5). The similar $\mathrm{pK}_a$ values for terephthalic acid and phthalic acid monomethyl ester, neither of which can form an intramolecular H bond in its anionic form, also suggest that the para-substituent constant provides a reasonable estimate for the intrinsic effect of an ortho-substituent (5, 10).

Determination of Bronsted Slopes for H Bonding. Bronsted slopes for the H bond in SA monomers were determined from linear least squares fit to plots of log $K_{\text{HB}}^\text{H}$ versus $\Delta \mathrm{pK}_a$.

The slope is expected to provide information about properties of the H bond because a homologous series of compounds was used. Though a small systematic error in the estimated intrinsic effect of the ortho-OH or COOH group is possible, it is expected to be constant and not to affect the dependence of log $K_{\text{HB}}$ on $\Delta \mathrm{pK}_a$ (i.e., the slope). To avoid compounds that might show deviant behavior, additional substituents ortho to either the COOH or OH group were not used, nor were charged substituents included (7–9).

It should be noted that the Brønsted coefficient is typically defined as the slope of the linear dependence of log $K_{\text{HB}}^\text{H}$ on the $\mathrm{pK}_a$ values of the donor or acceptor, while the other H bonding group remains constant (11–14). For the intramolecular H bond in SA monomers, the $\mathrm{pK}_a$ values of both the donor and acceptor are changed by the substituent as $\Delta \mathrm{pK}_a$ is varied. Some deviation from a simple linear correlation might therefore be anticipated. Such deviations are expected to be small, however, because the intrinsic $\mathrm{pK}_a$ of the COOH group vary over a limited range and Bronsted slopes have small dependences on the $\mathrm{pK}_a$ of the donor or acceptor (10–14).

NMR Spectroscopy. All measurements were performed at 400 MHz, 15–20°C. Solutions of substituted SAs or their triethylammonium salts in DMSO-$d_6$ (≈0.2 M) were used in
The equilibrium for formation of the intramolecular H bond between the hydroxyl proton and the carboxylate in a series of substituted salicylate monooanions was investigated as a function of \( \Delta \Delta pK_a \), the difference in \( pK_a \) between the H bond donor and acceptor.

Experimental Design. The equilibrium for formation of the intramolecular H bond (K\( ^{III} \)) was estimated according to Scheme II. Stabilization of the salicylate monooanion by the H bond renders deprotonation of the carboxylic acid more favorable, thereby decreasing its \( pK_a \) value (pK\( _{a\text{obsd}} \)). The decrease of the observed \( pK_a \) of the carboxylic acid relative to its intrinsic \( pK_a \) value (pK\( _{a\text{in}} \)) provides a measure of the strength of the H bond (Scheme IIa, \( \log K^{III} = pK_{a\text{int}} - pK_{a\text{obsd}} \)). The intrinsic \( pK_a \) of the carboxylic acid group refers to its \( pK_a \) value expected in the absence of H bonding (Scheme IIa, K\( _{a\text{in}} \)), which accounts for perturbation of the \( pK_a \) of benzoic acid by the polar and resonance effects of the ortho- OH substituent (see Materials and Methods).

A substituent at the 4- or 5-position of SA provides a means of varying the relative \( pK_a \) values of the donor and acceptor (\( \Delta pK_a \)). This is because deprotonation of the hydroxyl group has a steeper dependence on substituents than does the carboxylic acid group (\( \rho \) is \( \approx 2.2 \)-fold larger for ionization of phenols than benzoic acids, see Materials and Methods). In addition, the substituent has different substituent constants for ionization of the carboxylic acid and hydroxyl groups because it is para to one group and meta to the other (\( \sigma_p \) versus \( \sigma_m \)). This allows a \( \Delta pK_a \) range of 4.5 and 1.9 to be spanned in DMSO and water, respectively (Table 1).

Changes in H Bond Strength with Changes in \( \Delta pK_a \) in DMSO and Water. The pK\( _{a\text{obsd}} \), pK\( _{a\text{in}} \), and log K\( ^{III} \) values for each compound are summarized in Table 1. The free energy of H bonding (\( \Delta G^{III} \)) changes from \(-6.6 \) to \(-9.0 \) kcal/mol as \( \Delta pK_a \) decreases from 4.9 to 0.1 in DMSO. For the same series of compounds, \( \Delta G^{III} \) varies by only 0.2 kcal/mol, from \(-2.2 \) to \(-2.4 \) kcal/mol, with a change in \( \Delta pK_a \) of \(-2 \) units in water. Fig. 1 shows the dependence of log K\( ^{III} \) on the \( \Delta pK_a \) values of the H bond. To allow a direct comparison of the magnitude of changes in H bond strength that accompanies a given change in the charge distribution of donor/acceptor, a common scale of \( \Delta pK_a \) in water was used for the H bond in both media. The Brønsted slope \( \beta \), which describes the linear dependence of log K\( ^{III} \) on \( \Delta pK_a \), is 0.73 and 0.05 for the H bond in DMSO and water, respectively (Fig. 1). The larger Brønsted slope in DMSO than in water indicates that there is a greater increase in H bond strength in a nonaqueous media than aqueous solution as the charge density on the donor/acceptor is increased. This is more clearly depicted in Fig. 2, in which the equilibrium of H bonding in DMSO and water were directly compared: K\( ^{III} \) increases by two orders of magnitude in DMSO while it varies by \( \approx 2 \)-fold in water. The slope of log K\( ^{III} \) in DMSO versus log K\( ^{III} \) in water, which represents the different degree of strengthening of the H bond in the two media, is \( \approx 15 \). Though the steep slope precludes a precise estimate of its absolute value, it is substantially greater than the slope of unity (Fig. 2, dashed line) that would be observed if H bond strength has the same dependence on \( \Delta pK_a \) in DMSO and water.

H Bonds Near Matched \( pK_a \). The chemical shifts of the hydroxyl protons in SA monooanions are 4–5 ppm more downfield than those in the acid species, and fall in a range of 14-16 ppm (Table 2). In addition, the isotope fractionation factor for the hydroxyl proton in SA monooanion is 0.84 ± 0.03.

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\[^{8}\]The steeper dependence of log K\( ^{III} \) upon \( \Delta pK_a^{\text{water}} \) in DMSO can be broken down into two components. (i) The pK\( _a \) scale in DMSO is expanded: for ionization of phenols and benzoic acids, a change in \( \Delta pK_a \) of 1 in water corresponds to a change in \( \Delta pK_a \) of \( \approx 2.4 \) in DMSO (Table 1 and refs. 2–4). (ii) A steeper dependence of H bond strength on the proton affinity of the donor and acceptor: a plot of log K\( ^{III} \) versus \( \Delta pK_a^{\text{DMSO}} \) for the H bond in salicylates yields a Brønsted slope of 0.30 (not shown), whereas the slope is 0.05 for the corresponding H bonds in water (Fig. 1).

\[^{9}\]The Brønsted slope of 0.05 for H bonding in SA monooanions in water is similar to the value of 0.04–0.05 calculated from the Hine equation (Eq. 3), using a \( \tau \) value of 0.01 in water (11–14).

\[ \beta = \tau(pK_a^{\text{COOH}} - pK_a^{\text{H}_2\text{O}}). \]  

This equation is based on an electrostatic model of H bonding and describes H bonding in water as the competition between H bonding between solutes versus H bonding to water (11–14).

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\[^{10}\]H bonding between the COOH and OH groups may also be present in the neutral acids. This would increase the observed \( pK_a \) values of the carboxylic acids, leading to underestimate of the strength of the H bond in the monooanion. This effect is expected to be small, however, because the H bonds involving neutral species in the acid form are generally much weaker than those involving charged species in the anionic form (10–14, 16–25).
These properties are consistent with general criteria suggested for "short, strong" or low-barrier H bonds (for simplicity, LBHB is used to refer to all classes of "special" H bonds; e.g., refs. 10 and 26–33). It has been suggested that these properties arise from the development of covalent character in H bonds between donor and acceptor with matched or similar $p_K$ values, and that these H bonds may be especially strong due to additional stabilization from the covalent character (26–33).

![Diagram of covalent bond](https://example.com/diagram.png)

**COOH**

**CH$_3$OH**

**Diagram 3**

**Discussion**

The energetics of H bonds as a function of $\Delta p_K$ in nonaqueous and aqueous media provides a crude model for the changes in H bond strength accompanying charge rearrangements in the course of enzymatic and solution reactions, respectively. Results from this and previous studies suggest that the strengthening of the H bond is greater at a nonaqueous enzyme active site than in water; this could be used by enzymes to achieve a rate enhancement relative to the solution reaction.

**Larger Increases of H Bond Strength in Nonaqueous Media: Implications for Enzymatic Catalysis.** In the course of a reaction, charge accumulation on a substrate functional group could lead to the strengthening of H bonds to this group. However, rate enhancement relative to the solution reaction requires that the strengthening of the H bond to enzymatic catalytic groups be greater than that to water. The steeper dependences of H bond strength on $\Delta p_K$ generally observed in organic solvents and the gas phase than in water suggested that this is possible (10, 11, 16–25, 36). However, we were aware of no direct comparison of these dependences in nonaqueous and aqueous media.

We have compared the changes of H bond strength in DMSO and water for a series of substituted salicylate monoanions. There is a greater strengthening of the H bond with changes in the $p_K$ values of donor/acceptor in DMSO than in water (Figs. 1 and 2). Analogously, a greater increase in H bond strength accompanying charge rearrangements at a nonaqueous active site than in water could contribute to rate enhancement by enzymes. This is illustrated in Fig. 3 for the example of the TIM reaction. The H bond to the substrate carbonyl oxygen from either the enzymatic histidine or water is strengthened as charge relocalizes in the course of the reaction. The strengthening of the H bond would be greater in the nonaqueous enzymatic interior than in water (\(\Delta G^E > \Delta G^\text{DMSO}\)), provided that DMSO provides a reasonable, albeit crude, mimic of the active site environment (see below). The different degree of strengthening of the H bond provides rate enhancement for the enzymatic reaction relative to the solution reaction (\(\Delta G^* = \Delta G^E - \Delta G^\text{DMSO}\)).

If the linear dependences of H bond strength upon $\Delta p_K$ for the salicylates in water and DMSO [slopes of 0.05 and 0.73, respectively (Fig. 1)] were to hold for the solution and active site H bonds, respectively, the interaction shown in Fig. 3 would be expected to provide a rate enhancement of \(10^{98}\) fold for the enzymatic reaction (\(\Delta p_K\text{water} \approx 10; \text{rate enhancement} = 10^{\Delta p_K \times 10^9} = 10^{2 \times 10^9} = 10^{98}; \Delta G^* \approx 9\)).

![Diagram of potential catalysis](https://example.com/diagram.png)
The very crude estimate does not take into account all the factors that contribute to the change in the strength of this H bond; nevertheless it suggests that substantial rate enhancements can be obtained from steep dependences of H bond strength upon $\Delta pK_a$ at active sites and large changes in the $pK_a$ of the interacting groups.

These results support recent suggestions that H bond strength can undergo large changes in the course of an enzymatic reaction (e.g., 29–33). However, previous proposals emphasized the change in the nature of H bonds near matched $pK_a$ values to obtain a “special” resonance stabilization; the results presented here and elsewhere provide no indication of such a special energetic contribution (refs. 10, 16–25, and 34–36; see “H Bonds Near Matched $pK_a$” in Results). The steep dependence of H bond strength appears to extend beyond the region near matched $pK_a$ values where there may be significant covalent bond character (Fig. 1; refs. 16–25). We therefore suggest that a large increase in H bond strength upon charge rearrangement can be achieved in enzyme active sites for H bonds that are predominantly electrostatic, and we discuss the results herein in terms of a simple electrostatic model for H bonding (see below).

It remains possible that a special energetic contribution from the covalent character of LBHBs may be observed in other systems. Even in the absence of a special contribution to the free energy of H bond formation, an LBHB might allow a greater precision of positioning within an active site because it is shorter and covalent in character. It is also conceivable that enzymes position functional groups closely in the ground state to create destabilization that is relieved in the transition state by the ability to form a short hydrogen bond (C. S. Cassidy, J. Lin, and P. A. Frey, personal communication).

Do Enzymes Exhibit Large Increases in H Bond Strength Upon Charge Rearrangement? A recent investigation of the stability of a series of Staphylococcal nuclease mutants suggests that the enzymatic interior can provide an environment for large Brønsted slopes of H bonding (37). In these proteins, the acidity of a tyrosine hydroxyl group (Tyr-27) was varied by fluoro-substitutions on the aromatic ring (2-F,3-F, or 2,3,5,6-F$_4$); this provides a protein model for the effects of charge rearrangement. As the tyrosine hydroxyl becomes more acidic, its H bond to Glu-10 becomes stronger, stabilizing the folded protein (Fig. 4A; $K_{f}^{[H]}$); on the other hand, its H bond to water would also be strengthened, stabilizing the unfolded protein (Fig. 4A; $K_{u}^{[H]}$). The observed stability of the protein (Fig. 4A, $K_{folding}^{[H]}$) increases with increasing acidity of the tyrosine hydroxyl. This indicates that the strengthening of the H bond between the X-tyr$^{OH}$-glu$^{COO^-}$ pair is greater than the strengthening of the H bond to water (Fig. 4B, $\Delta \log K_{folding}^{[H]} = \Delta \log K_{f}^{[H]} - \Delta \log K_{water}^{[H]}$). The slope of the plot of $\log K_{folding}^{[H]}$ versus the $pK_a$ of the tyrosine hydroxyl was 0.35–0.75 (37). This suggests that the Brønsted slope is 0.35–0.75 larger for the X-tyr$^{OH}$-glu$^{COO^-}$ H bond on the enzyme than for the X-tyr$^{OH}$-water H bond in aqueous solutions (Fig. 4B).

Origins of the Larger Brønsted Slopes of H Bonding in Nonaqueous Environments. The energetics of most H bonds can be described by a simple electrostatic model (Eqs. 1 and 2), in which H bond energy ($E^H$) is dominated by the Coulombic interaction

$$E^H = -\frac{k e_{eff}^f \times q_1 q_2}{r}$$

[1]

$$\Delta E^H = -\frac{k e_{eff}^f \times (\Delta q_1) q_2}{r}$$

[2]

between the partial effective charges on the donor and acceptor (q1 and q2, respectively), with an interaction coefficient that depends on the effective dielectric of the media ($e_{eff}$) and the distance separating the partial charges (r). This model, though crude, provides a useful conceptual tool for considering factors that contribute to the greater sensitivity of H bond strength to charge rearrangements in nonaqueous media and enzymatic interior relative to water.

The enzyme active site can be considered as an environment of low effective dielectric (38–40). Such an environment would increase the change in H bond strength accompanying a charge.

Fig. 4. A large Brønsted slope for H bonding in proteins is inferred from folding studies of Staphylococcal nuclease mutants. (A) Thermodynamic analysis depicting the effect of changing the strength of the H bond donor, the substituted tyrosine hydroxyl, on the stability of the protein. The folding equilibrium of a hypothetical non-H-bonded species ($K_{folding}^{[H]}$) is used to dissect the effects from H bonding. (B) Schematic depiction of the dependences of H bonding and folding equilibria on the $pK_a$ value of substituted tyrosines. As the tyrosine hydroxyl becomes more acidic, the strengthening of its H bond to the enzymatic glutamate and to water stabilizes the folded and unfolded protein, respectively. The slope of the plot of $\log K_{folding}^{[H]}$ versus $pK_a$ is the difference between the slopes of plots of $\log K_{f}^{[H]}$ versus $pK_a$ and $\log K_{water}^{[H]}$ versus $pK_a$. This follows from the thermodynamic relationship shown in A, which gives $\Delta \log K_{folding}^{[H]} = \Delta \log K_{f}^{[H]} - \Delta \log K_{water}^{[H]}$ (i.e., the greater strengthening of the H bond on the protein results in a change in the observed stability of the protein. Note that $\Delta \log K_{f}^{[H]} = 0$ by definition because the folding equilibrium between the non-H-bonded species does not depend on the strength of the H bond donor.

The observed stability of the proteins at pH 7.0 as a function of the aqueous $pK_a$ of substituted tyrosines gives a Brønsted slope of 0.35–0.75 (37). The actual Brønsted slope for the H bond is likely to be larger because tetrafluorotrotyrosine is predominantly deprotonated in solution at pH 7.0 ($pK_a \approx 5.3$ (37)). The folded protein with the deprotonated tetrafluorotrotyrosine is expected to be less stable than those with the protonated form because of electrostatic repulsion between the anionic glutamate and hydroxylate of tetrafluorotrotyrosine. This presumably leads to an underestimate of the stability of the protein with the neutral tetrafluorotrotyrosine. Correcting for the fraction of deprotonated species at pH 7.0, assuming that all of the folded proteins contain the protonated tetrafluorotrotyrosine, yields a Brønsted slope of 0.75. This provides an estimate for the upper limit of the Brønsted slope.
remediation [Eq. 2, \((\Delta E^\text{H}/\Delta q) \approx (1/\varepsilon_o)\)]. The effective dielectric is used as a simplified parameter to describe the ability of the environment to stabilize charged ions and dipoles: the better this stabilization is accomplished, the less the energetic consequences of charge development. The effective dielectric is distinct from the bulk dielectric constant; it can account for the impact of the molecular properties of the local environment on interactions between adjacent groups, such as H bonds (41–45).

What are the molecular properties that contribute to the lower effective dielectric of nonaqueous solvents and enzymatic active sites relative to water? Water is a highly polar molecule that can effectively stabilize developing charges on a solute, thereby attenuating the observed increase in H bond strength between solutes. In addition, the ability of water molecules to rearrange further decreases the energetic difference between stronger and weaker solute/solute interactions. In contrast, the enzymatic interior is replete with hydrophobic residues, and the rigidity of the active site limits rearrangement of polar groups and bound water that are present. Similarly, DMSO is not capable of providing effective solvation to negative charges such as those in salicylicates because it contains no effective H bond donor (see ref. 6). The larger size of DMSO also limits its ability to rearrange in the first solvation layer in response to charge redistribution. DMSO may therefore provide a crude mimic for the environment in an enzymatic interior, despite its high bulk dielectric constant. In general, larger Bronsted slopes for H bonds are observed in nonaqueous solvents than in water (10, 11, 16–25, 36).

The ability of enzyme active sites to provide functional groups that are stronger H bond donor/acceptor than water can also contribute to catalysis (46). Larger Bronsted slopes of H bonding for stronger donors or acceptors have been observed in previous model studies (12–14). This is consistent with expectations from the electrostatic model of H bonding, in which the change in H bond strength accompanying a change in the partial charge of the donor, for instance, is predicted to increase with increasing partial charges on the acceptor [Eq. 2, \((\Delta E^\text{H}/\Delta q) \approx \epsilon \eta \) (12–14, 46)]. This factor is not present in this model study, but may contribute in part to the larger Bronsted slope observed with Staphylococcal nuclease (see above).

What Provides the Driving Force for H Bond Formation in Nonaqueous Media? The equilibria for formation of the H bond in S/A monooanions are 10^4-fold more favorable in DMSO than in water (Fig. 1). This does not imply, however, that the H-bonded species are more stable in nonaqueous media than in aqueous solutions. As the equilibrium for H bond formation depends on the relative stability of the bonded and nonbonded species, the more favorable H bonding in nonaqueous media may rather reflect the high instability of isolated charges and dipoles in these environments relative to water, which is lessened by H bond formation. Binding interactions of an enzyme may be used to pay for the energetic penalty of desolvating the H bonding groups and fixing them in the low dielectric active site (47, 48), thereby allowing the enzyme to maximally discriminate between the different charge distributions of the ground state and transition state. The H bond donor and acceptor in the S/A monooanions are fixed with respect to each other by covalent interactions, so that this system provides a model for the energetic forces of H bonding between groups that are already positioned at the enzyme active site by binding interactions. Biochemistry: Shan and Herschlag