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

(hydrogen bonding/active site environments)

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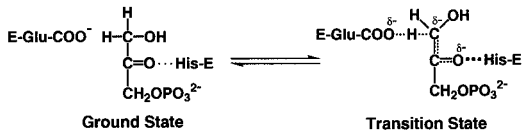
ABSTRACT **The equilibrium for formation of the intramolecular hydrogen bond (K^{HB}) in a series of substituted salicylate monoanions was investigated as a function of ΔpK_a, the difference between the pK_a values of the hydrogen bond donor and acceptor, in both water and dimethyl sulfoxide. The dependence of log K^{HB} upon ΔpK_a 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 rearranges 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.



Scheme **I**

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_a of this group. [The pK_a of the carbonyl oxygen in the ground state is estimated to be −2 to 0; the pK_a 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_a 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-*tert*-butyl-4-nitrophenol and 9-carboxymethylfluorene were synthesized from 2,6-di-*tert*-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 P_2O_5 for >24 hr prior to use. Compounds with purity <98% were further purified by recrystallization.

Determination of pK_a^{obsd} in DMSO. Observed pK_a values (pK_a^{obsd}) 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-*tert*-butyl-4-nitrophenol, and 9-carboxymethylfluorene [pK_a values of 5.12, 7.30, and 10.35 in DMSO, respectively (2, 3)]. Each pK_a in Table 1 was measured with two indicators whose pK_a values bracket that of the compound of interest. Standard deviations in the pK_a^{obsd} values from independent determinations varied from ±0.02 to ±0.14, with an average standard deviation of ±0.06.

Determination of pK_a^{obsd} in Water. Observed pK_a 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_a 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 K^{HB} has a small dependence on ΔpK_a in water. However, use of the same set of buffers for the pK_a determination of all compounds results in less scatter in the plots of log K^{HB} (water) versus ΔpK_a and log K^{HB} (DMSO) versus log K^{HB} (water), presumably because of an increase in the precision of the data.

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Abbreviations: DMSO, dimethyl sulfoxide; TIM, triosephosphate isomerase; SA, salicylic acid; LBHB, low-barrier H bonds.

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Table 1. Formation constants and ΔpK_a values of the H bond in substituted SA monoanions in DMSO and water

Compound	pK_a^{obsd}*		pK_a^{int}†		ΔpK_a*		log K^{HB}‡	
	DMSO	H_2O	DMSO	H_2O	DMSO	H_2O	DMSO	H_2O
4-OH-SA (**1**)	7.1	3.2	13.7	5.0	0.1	3.0	6.6	1.8
4-Br-SA (**2**)	5.5	2.7	11.9	4.4	0.8	3.3	6.4	1.7
4-OMe-SA (**3**)	7.1	3.2	13.1	4.9	0.9	3.4	5.9	1.7
5-Br-SA (**4**)	5.3	2.5	11.5	4.2	1.7	3.7	6.2	1.7
5-Cl-SA (**5**)	5.4	2.5	11.5	4.2	2.0	3.8	6.1	1.8
4-Me-SA (**6**)	7.0	3.0	12.7	4.7	2.2	3.9	5.8	1.7
SA (**7**)	6.6	2.9	12.4	4.6	2.2	3.9	5.8	1.7
5-F-SA (**8**)	5.8	2.5	11.6	4.2	2.7	4.1	5.8	1.7
5-Me-SA (**9**)	6.8	3.0	12.5	4.6	2.9	4.2	5.7	1.7
5-OMe-SA (**10**)	6.6	2.8	12.1	4.5	3.1	4.3	5.5	1.7
5-OH-SA (**11**)	7.1	2.8	12.1	4.5	4.5	4.9	5.0	1.6

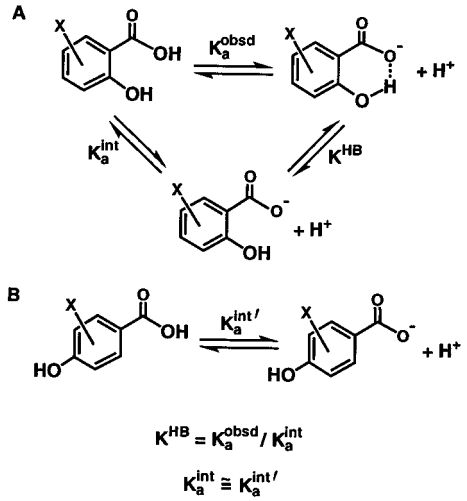
Only one significant number is reported for the pK_a values for clarity, though the more precise pK_a values were used to calculate log K^{HB} and ΔpK_a.

*Determined as described in *Materials and Methods.*

†Values listed are the intrinsic pK_a values of the COOH groups, determined as described in *Materials and Methods*. The intrinsic pK_a values of the OH groups are not listed for simplicity, though they were used to calculate the ΔpK_a values. These values can be obtained from $pK_a^{\Phi OH,int} = pK_a^{\Phi COOH,int} + \Delta pK_a$ (see *Materials and Methods*).

‡Obtained from the relationship: $\log K^{HB} = \log(K_a^{obsd}/K_a^{int}) = pK_a^{int} - pK_a^{obsd}$, derived from Scheme **IIA**.

Determination of pK_a^{int} and ΔpK_a. The "intrinsic" pK_a (pK_a^{int}; see also *Results*) represents the 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 **IIB**).



Scheme **II**

ΔpK_a represents the difference in the intrinsic pK_a values of the H bond donor and acceptor. Thus, the pK_a^{int} values of both the COOH and OH groups were determined to obtain the ΔpK_a value for the H bond in each compound.

The pK_a^{int} value was estimated as the pK_a of the functional group in a corresponding compound with the OH group *para* to the COOH instead of *ortho*, so that no intramolecular H bond can form (Scheme **IIB**). For salicylic acid (SA), the intrinsic pK_a of the COOH group [$pK_a^{\Phi COOH,int}$(SA)] was estimated as the pK_a value of 4-hydroxybenzoic acid [12.4 (6)]. For SAs with a substituent X at the 4- or 5-position (X-SA), the intrinsic pK_a of the COOH group was calculated from: $pK_a^{\Phi COOH,int}$ (X-SA) = [$pK_a^{\Phi COOH,int}$(SA) − $\sigma_X * \rho^{\Phi COOH}$], where σ_X is the Hammett constant describing the electron-withdrawing ability of the substituent X and $\rho^{\Phi COOH}$ is the slope of the linear dependence of pK_a on the σ value of the substituent for benzoic acids. The intrinsic pK_a of the OH group of SA was obtained from the relationship: $pK_a^{\Phi OH,int}$(SA) = $pK_a^{\Phi OH} - \sigma_{p\text{-COOH}} * \rho^{\Phi OH}$, where $\sigma_{p\text{-COOH}}$ is the Hammett constant of a *p*-COOH substituent and $\rho^{\Phi OH}$ is the slope of the linear dependence of pK_a on the σ value of the substituent for phenols. The intrinsic pK_a of the OH group in substituted salicylates was calculated from: $pK_a^{\Phi OH,int}$(X-SA) = [$pK_a^{\Phi OH,int}$(SA) − $\sigma_X * \rho^{\Phi OH}$]. The Hammett constants σ_m and σ_p were used for the ionization of COOH groups, since these constants are based on the 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 σ_- scale was used for the pK_a values of the OH groups (7, 8). The effect of a *p*-COOH substituent on the pK_a of the phenol hydroxyl (i.e., σ_-) cannot be obtained because the COOH group deprotonates before the OH group does; the σ_- value of *p*-COOR 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^{HB} versus ΔpK_a or the conclusions derived herein (see also below). The ρ 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 pK_a values of carboxylic acids in SAs arise from the H bonding interactions in their monoanions in both solvents, and that the determination of pK_a^{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 pK_a values for SAs relative to the corresponding *para*-compounds can be attributed to H bonding. In addition, comparison of the 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 pK_a of benzoic acids by <1 pK unit in both solvents (see ref. 6 for a summary of the relevant pK_a values in DMSO; the corresponding aqueous pK_a values can be found in ref. 5). The similar 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 Brønsted Slopes for H Bonding. Brønsted slopes for the H bond in SA monoanions were determined from linear least squares fit to plots of log K^{HB} versus ΔpK_a^{water}. 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^{HB} on ΔpK_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^{HB} on the 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 monoanions, the pK_a values of both the donor and acceptor are changed by the substituent as ΔpK_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 pK_a of the COOH group vary over a limited range and Brønsted slopes have small dependences on the 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

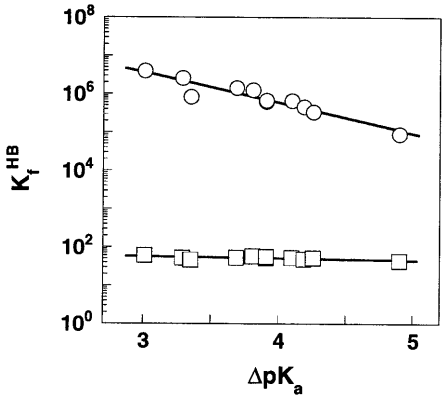


FIG. 1. The dependence of log K^{HB} upon ΔpK_a^{water} in DMSO (○) and H_2O (□) for the H bond in substituted SA monoanions. The Brønsted slopes are 0.05 and 0.73 in water and DMSO, respectively. A common ΔpK_a scale in water was used to allow a direct comparison of the magnitude of changes in H bond strength in the two solvents. Data are from Table 1.

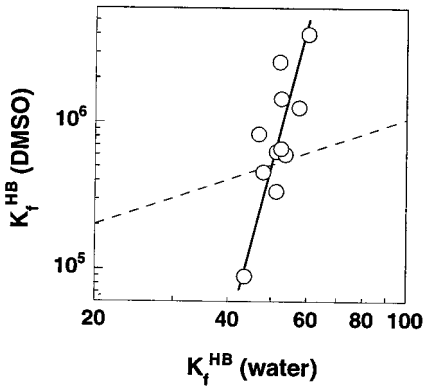


FIG. 2. A greater increase in H bond strength accompanying changes in the charge density of donor/acceptors in DMSO than in water. The logarithm of equilibrium constants for H bonding in DMSO are plotted against those in water for substituted salicylates (note the difference in scales). The solid line represents a least squares fit of the data and gives a slope of 15. The dashed line with a slope of unity, which represents the correlation expected if the strengthening of the H bond were the same in the two media, is shown for comparison. Data are from Table 1.

chemical shift measurements. The isotope fractionation factor for the H-bonded proton in SA monoanion was measured by NMR spectroscopy as described (10, 15).

RESULTS

The equilibrium for formation of the intramolecular H bond between the hydroxyl proton and the carboxylate in a series of substituted salicylate monoanions was investigated as a function of ΔpK_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^{HB}) was estimated according to Scheme **II**. Stabilization of the salicylate monoanion by the H bond renders deprotonation of the carboxylic acid more favorable, thereby decreasing its pK_a value (pK_a^{obsd}). The decrease of the observed pK_a of the carboxylic acid relative to its intrinsic pK_a value (pK_a^{int}) provides a measure of the strength of the H bond (Scheme **IIA**, log $K^{HB} = pK_a^{int} - pK_a^{obsd}$).‡ 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^{int}), 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 (ΔpK_a). This is because deprotonation of the hydroxyl group has a steeper dependence on substituents than does the carboxylic acid group (ρ is ≈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 (σ_p versus σ_m). This allows a ΔpK_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 ΔpK_a in DMSO and Water. The pK_a^{obsd}, pK_a^{int}, and log K^{HB} values for each compound are summarized in Table 1. The free energy of H bonding (ΔG^{HB}) changes from −6.6 to −9.0 kcal/mol as ΔpK_a decreases from 4.9 to 0.1 in DMSO. For the same series of compounds, ΔG^{HB} varies by only 0.2 kcal/mol, from −2.2 to −2.4 kcal/mol, with a change in ΔpK_a of ≈2 units in water. Fig. 1 shows the dependence of log K^{HB} on the ΔpK_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 ΔpK_a in water was used for the H bond in both media.§ The Brønsted slope β, which describes the linear dependence of log K^{HB} on ΔpK_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^{HB} increases by two orders of magnitude in DMSO while it varies by <2-fold in water. The slope of log K^{HB} in DMSO versus log K^{HB} in water, which represents the different degree of strengthening of the H bond in the two media, is ≈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 ΔpK_a in DMSO and water.

H Bonds Near Matched pK_a. The chemical shifts of the hydroxyl protons in SA monoanions 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 monoanion is 0.84 ± 0.03.

‡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 monoanion. 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).

§The steeper dependence of log K^{HB} upon ΔpK_a^{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 ΔpK_a of 1 in water corresponds to a change in ΔpK_a of ≈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^{HB} versus ΔpK_a^{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).

¶The Brønsted slope of 0.05 for H bonding in SA monoanions in water is similar to the value of 0.04–0.05 calculated from the Hine equation (Eq. **3**), using a τ value of 0.01 in water (11–14).

$$\beta = \tau(pK_a^{COOH} - pK_a^{H_3O^+}). \quad [3]$$

This equation is based on a 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).

Table 2. Chemical shifts of the H-bonded proton in SA monoanions in DMSO

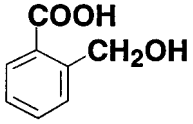
Compound	ΔpK_a^{DMSO}*	δ_H (acid)†	δ_H (anion)†
4-OMe-SA (**3**)	0.9	11.5	15.6
5-Cl-SA (**5**)	2.0	12.2	15.8
SA (**7**)	2.2	11.4	16.3
5-F-SA (**8**)	2.7	11.6	15.2
5-Me-SA (**9**)	2.9	11.3	14.9
5-OMe-SA (**10**)	3.1	11.0	14.6

*From Table 1.
†Determined as described in *Materials and Methods.*

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 acceptors with matched or similar pK_a values, and that these H bonds may be especially strong due to additional stabilization from the covalent character (26–33).

Unusual IR spectra, which may also reflect the covalent character of these H bonds, are observed in organic solvents for H bonds between groups with $\Delta pK_a^{H_2O} < 1$–2 units in organic solvents (e.g., refs. 34–36). However, the energetics of H bonds investigated to date provide no indication of a steeper increase in H bond strength in this ΔpK_a region that might be expected from a special energetic contribution from covalent character. For example, the H bond in substituted salicylates investigated herein exhibits a linear dependence of log K^{HB} on ΔpK_a over a range of $\Delta pK_a^{H_2O}$ values of 1.9, without any upward curvature near matched pK_a in DMSO (Fig. 1; ΔpK_a^{DMSO} ranges from 0.1 to 4.5; Table 1). There is also no curvature in Brønsted slopes for H bonding between substituted phenols and phenolates in tetrahydrofuran (THF), over a $\Delta pK_a^{H_2O}$ range from 0 to 3.1 units (10).

The following observation further suggests that the steep dependence of H bond strength on ΔpK_a persists even at large values of ΔpK_a, where covalent contributions to the H bond are expected to be minimal. The energy of the H bond between the CH_2OH and COO^- groups in 2-hydroxymethylbenzoic acid (**12**) was estimated to be 2.3 kcal/mol in DMSO by the procedure outlined in Scheme **II**. This compound exhibits a pK_a^{obsd} value of 9.6 in DMSO, which is 1.7 units lower than the pK_a^{int} value of 11.3 estimated from *p*-hydroxymethylbenzoic acid. This H bond has a ΔpK_a of ≈10 in water [pK_a^{int} (COOH) = 4.2 and pK_a^{int} (OH) ≈14 in water (5); ΔpK_a in DMSO is estimated to be >20 from the ΔpK_a value in water and the 2.4-fold larger ρ value for ionizations in DMSO relative to water (2–4)]. Despite this large ΔpK_a, the H bond strength for **12** falls on the linear Brønsted plot in Fig. 1 when this line is extended to $\Delta pK_a = 10$ (there is a positive deviation in log K^{HB} of 0.2 from the extrapolated line). These results are consistent with a continued linear decrease in H bond strength over a wider ΔpK_a range with no significant change in slope. Though this compound is not homologous to the salicylates, we expect the additional methylene group will have only a small effect on the H bond strength.



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DISCUSSION

The energetics of H bonds as a function of ΔpK_a 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 ΔpK_a 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 dependencies 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 pK_a values of donor/acceptors 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 ($\Delta\Delta G^E > \Delta\Delta G^{soln}$), 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 ($\Delta\Delta G^{\neq} = \Delta\Delta G^E - \Delta\Delta G^{soln}$).

If the linear dependences of H bond strength upon ΔpK_a 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 $\approx 10^6$ fold for the enzymatic reaction [$\Delta pK_a^{water} \approx 10$; rate enhancement = $10^{\Delta pKa \times \Delta\beta} = 10^{10\times(0.73-0.05)} = 6 \times 10^6$; $\Delta\Delta G^{\neq} \approx 9$

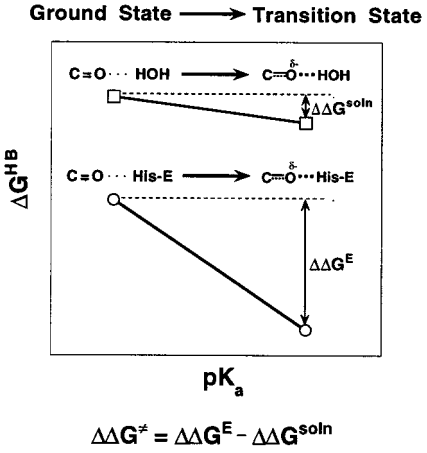

FIG. 3. Diagrammatic illustration of potential catalysis that can be obtained from a greater strengthening of H bonds accompanying charge rearrangements at a nonaqueous enzymatic active site than in water, using the example of the TIM reaction. The pK_a of the substrate carbonyl oxygen increases during the course of reaction, so that ΔpK_a between this oxygen atom and the enzymatic or solution H bond donor (His-E or HOH, respectively) decreases. This leads to an increase in H bond strength. This increase would be greater at the enzymatic active site ($\Delta\Delta G^E$) than in solution ($\Delta\Delta G^{soln}$) because of the greater Brønsted slopes in nonaqueous environments. The amount of catalysis obtained from this strategy, relative to the solution reaction, is $\Delta\Delta G^{\neq} = \Delta\Delta G^E - \Delta\Delta G^{soln}$.

kcal/mol]. This 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 ΔpK_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 at 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).

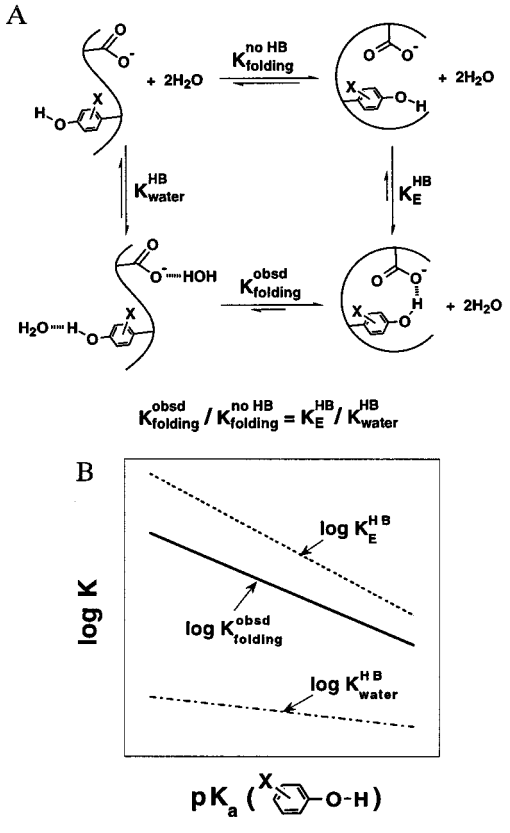

FIG. 4. A large Brønsted slope for H bonding in proteins is inferred from folding studies of *Staphylococcal* nuclease mutants. (*A*) Thermodynamic analysis depicting the effect of changing the strength of the H bond donor, the substituted tyrosine hydroxyl, on the stability of the protein. The folding equilibrium of a hypothetical non-H-bonded species ($K_{folding}^{no\ HB}$) 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 $K_{folding}^{obsd}$ versus pK_a is the difference between the slopes of plots of log K_E^{HB} versus pK_a and K_{water}^{HB} versus pK_a. This follows from the thermodynamic relationship shown in *A*, which gives $\Delta\log K_{folding}^{obsd} = \Delta\log K_E^{HB} - \Delta\log K_{water}^{HB}$ (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 $\Delta\log K_{folding}^{no\ HB} = 0$ by definition because the folding equilibrium between the non-H-bonded species does not depend on the strength of the H bond donor.

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 rearrangment. As the tyrosine hydroxyl becomes more acidic, its H bond to Glu-10 becomes stronger, stabilizing the folded protein (Fig. 4*A*, K_E^{HB}); on the other hand, its H bond to water would also be strengthened, stabilizing the unfolded protein (Fig. 4*A*, K_{water}^{HB}). The observed stability of the protein (Fig. 4*A*, $K_{folding}^{obsd}$) 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. 4*B*, $\Delta\log K_{folding}^{obsd} = \Delta\log K_E^{HB} - \Delta\log K_{water}^{HB}$). The slope of the plot of log $K_{folding}^{obsd}$ 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. 4*B*).

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

$$E^H = \frac{k}{\varepsilon_{eff} r} \times q_1 q_2, \qquad [1]$$

$$\Delta E^H = \frac{k}{\varepsilon_{eff} r} \times (\Delta q_1) q_2 \qquad [2]$$

between the partial effective charges on the donor and acceptor (q_1 and q_2, respectively), with an interaction coefficient that depends on the effective dielectric of the media (ε_{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

‖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 (37). The actual Brønsted slope for the H bond is likely to be larger because tetrafluorotyrosine is predominantly deprotonated in solution at pH 7.0 [$pK_a \approx 5.3$ (37)]. The folded protein with the deprotonated tetrafluorotyrosine is expected to be less stable than those with the protonated form because of electrostatic repulsion between the anionic glutamate and hydroxylate of tetrafluorotyrosine. This presumably leads to an underestimate of the stability of the protein with the neutral tetrafluorotyrosine. Correcting for the fraction of deprotonated species at pH 7.0, assuming that all of the folded proteins contain the protonated tetrafluorotyrosine, yields a Brønsted slope of 0.75. This provides an estimate for the upper limit of the Brønsted slope.

redistribution [Eq. **2**, $(\Delta E^{H}/\Delta q_1) \propto (1/\varepsilon_{eff})$]. The effective dielectric is used as a simplified parameter to describe the ability of the environment to stabilize isolated charges 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 salicylates 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 Brønsted slopes for H bonds are observed in nonaqueous solutions than in water (10, 11, 16–25, 36).

The ability of enzyme active sites to provide functional groups that are stronger H bond donor/acceptors than water can also contribute to catalysis (46). Larger Brønsted 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**, $(\Delta E^{H}/\Delta q_1) \propto q_2$] (12–14, 46). This factor is not present in this model study, but may contribute in part to the larger Brønsted 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 SA monoanions 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 SA monoanions are fixed with respect to each other by covalent interactions, so that this system provides a model for the energetic effects of H bonding between groups that are already positioned at the enzyme active site by binding interactions.

Binding interactions can also be used to impose ground state interactions between the enzyme and the substrate that are unfavorable compared with their interactions with water (refs. 47–49 and references therein). This provides an additional way to maximize the difference between the ground state and transition state interactions, and thus the catalytic contribution of H bonds.

Positioning of substrates with respect to one another and with respect to catalytic active site residues, a catalytic mechanism that often utilizes H bonding, can lower the entropic barrier for reaction (47, 48). The concept of increasing the sensitivity of H bond strength to charge rearrangement at an enzymatic active site is conceptually distinct from catalysis via such induced intramolecularity. Nevertheless, the catalytic contributions from these mechanisms may be inextricably linked in that both mechanisms are effected through the rigidity and precise pre-organization of the active site, the energetic driving force for which is provided by folding of the protein and binding interactions of the substrate with the folded enzyme. That is, folding of the enzyme and its binding interactions with the substrate are responsible both for fixing the substrate in a low dielectric environment that maximizes the increase in H bond strength in the course of a reaction and for positioning reactants with respect to pre-aligned enzymatic catalytic groups that reduces the entropic barrier of a reaction (e.g., refs. 47, 48 and references therein).

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