The Energetics of Hydrogen Bonds in Model Systems: Implications for Enzymatic Catalysis

Shu-ou Shan, Stewart Loh, Daniel Herschlag*

Low-barrier or short, strong hydrogen bonds have been proposed to contribute 10 to 20 kilocalories per mole to transition-state stabilization in enzymatic catalysis. The proposal invokes a large increase in hydrogen bond energy when the pK_a values of the donor and acceptor (where K_a is the acid constant) become matched in the transition state (ΔpK_a = 0). This hypothesis was tested by investigating the energetics of hydrogen bonds as a function of ΔpK_a for homologous series of compounds under nonaqueous conditions that are conducive to the formation of low-barrier hydrogen bonds. In all cases, there was a linear correlation between the increase in hydrogen-bond energy and the decrease in ΔpK_a, as expected from simple electrostatic effects. However, no additional energetic contribution to the hydrogen bond was observed at ΔpK_a = 0. These results and those of other model studies suggest alternative mechanisms by which hydrogen bonds can contribute to enzymatic catalysis, in accord with conventional electrostatic considerations.

Enzymes can achieve enormous rate enhancements, which often require >20 kcal/mol of transition-state stabilization. Recent proposals to account for these large energies have invoked low-barrier hydrogen bonds [LBHBs (*1*)] (*2*, *3*). An example of this hypothesis, showing enzymatic catalysis by an LBHB in the mandelate racemase reaction, is presented in Fig. 1. It was proposed that an LBHB is formed when the pK_a of the enolate hydroxyl in the enolic transition state becomes matched with that of Glu^{317} on the enzyme, which stabilizes the transition state by ~20 kcal/mol; in contrast, the pK_a of the carboxyl proton in the ground state is not matched with that of Glu^{317}, so that the ground state would be stabilized by only 1 to 5 kcal/mol from a normal H bond (*2*, *3*). LBHBs have also been proposed to provide a large fraction of the catalytic power for many other enzymes, including triose phosphate isomerase, ribonucleases, enolase, aconitase, and citrate synthase (*2*, *3*). The observation of a highly deshielded proton (δ_H = 18 parts per million) between His^{57} and Asp^{102} in the catalytic triad of trypsin and chymotrypsin and the isotope effect on this chemical shift ($\delta_H - \delta_D$ = 1.0 ppm) have been suggested as evidence for an LBHB that contributes to catalysis by serine proteases (*4*).

The concept of LBHBs originated from the large formation energy of $FH\cdots F^-$ in the gas phase [~40 kcal/mol (*5*, *6*)] and its short bond length [2.26 Å (*7*)] in the solid state. Low isotope fractionation factors (0.5 to 1.0) and anomalous spectroscopic properties, such as highly downfield proton chemical shifts (16 to 22 ppm) and inverse isotope effects on proton chemical shifts and infrared vibrational frequencies, have been observed for a number of H bonds in organic solvents, including phthalate and maleate monoanions, proton sponges, and cyclic diamines (*7*). It has been suggested that when an H bond is formed between groups with similar pK_a's, orbital overlap takes place between their matched energy states, which results in a bond that has partial covalent character and unusual spectroscopic properties (*7–10*). This partial covalent character could provide an additional energetic contribution to the strength of an LBHB (*2–4*, *7–10*). As proposed, an LBHB formed at matched pK_a's would then be stronger than an H bond stabilized solely by electrostatic interactions.

The proposal that LBHBs can provide a special contribution to enzymatic catalysis can be experimentally tested by determining the H-bond energy as a function of ΔpK_a, the difference in pK_a between the donor and the acceptor. Simple electrostatic considerations predict that H-bond strength increases linearly with increasing acidity of the donor or increasing basicity of the acceptor, reaching its maximum at ΔpK_a = 0 (*8*, *11*, *12*). Any additional energetic contribution from covalent character in an LBHB would lead to the formation of an especially strong H bond at matched pK_a, resulting in a positive deviation at ΔpK_a = 0.

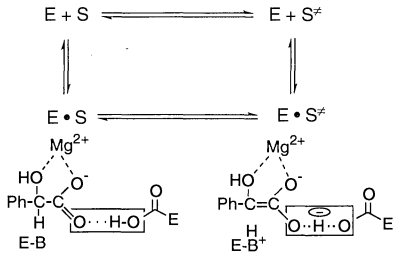


Fig. 1. A simplified illustration of the proposal of LBHB participation in enzymatic catalysis, showing proton abstraction catalyzed by mandelate racemase. An LBHB between the carboxyl oxygen and Glu^{317} on the enzyme would stabilize the enolate intermediate or transition state (*2*, *3*). Bond notation in boxes: (···) represents the normal, weak H bond in the ground state (E·S) with unmatched pK_a's; (··) and the intermediate position of the proton represent the strong LBHB formed in the transition state (E·S≠) when their pK_a's become matched. Ph, phenyl.

Previous work has provided no evidence for an extraordinarily strong H bond that forms specifically at ΔpK_a = 0. The gas-phase formation energy (ΔG_f and ΔH_f) of the asymmetrical $CH_3O^-\cdots HOH$ H bond is within ~5 kcal/mol of the symmetrical $HO^-\cdots HOH$ and $CH_3O^-\cdots HOCH_3$ H bonds (*13*). In addition, gas-phase studies of the association of F^- with various acids revealed a linear correlation between H-bond energy and ΔpK_a, without a deviation at ΔpK_a = 0 (*5*). Similarly, no deviation at ΔpK_a = 0 was observed in studies of H bonding in water and organic solvents (*12*, *14*). However, the studies cited above were not carried out to test the energetics of LBHBs and thus do not provide conclusive evidence against the LBHB proposal. LBHBs do not appear to form in aqueous solution (*2*, *3*, *7*, *10*), and the gas-phase and organic-solvent studies either lacked a data point for an H bond with perfectly matched donor and acceptor pK_a's or used compounds that do not represent a homologous series.

We therefore systematically investigated H-bond energy as a function of ΔpK_a for a series of substituted phthalate monoanions (X-PAs; Scheme 1) in dimethyl sulfoxide (DMSO). The H bond between the adjacent carboxyl groups of PA^- has been considered to be an example of an LBHB (*7*, *10*) on the basis of the 21-ppm chemical shift of the H-bonded proton, the negative

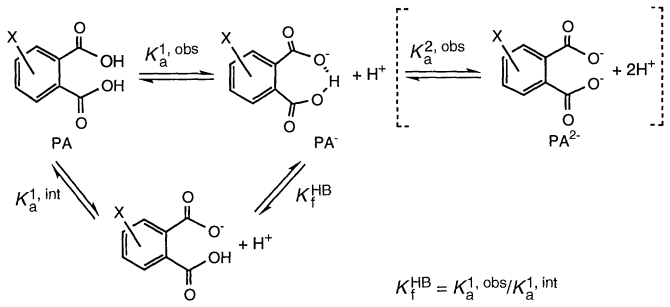
Table 1. Chemical shifts and isotope fractionation factors (Φ) of the H-bonded proton in substituted PAs in DMSO. All NMR measurements were performed at 400 MHz and 15° to 20°C. Values of ΔpK_a are from Table 2; values of Φ were determined as described in (*44*). Me, methyl; –, not determined.

Compound	ΔpK_a	δ_H (acid)	δ_H (monoanion)	Φ
PA	0	13.2	20.8 (21.0*)	0.56 ± 0.06
4-NO_2-PA	0.024	13.8	20.4	–
4-Me-PA	0.24	13.0	20.5	–
4-Cl-PA	0.38	–	20.5	0.51 ± 0.07
4-Br-PA	0.46	13.4	20.4	–

*From (*15*).

Department of Biochemistry, B400 Beckman Center, Stanford University, Stanford, CA 94305–5307, USA.

*To whom correspondence should be addressed. E-mail: herschla@cmgm.stanford.edu



$K_f^{HB} = K_a^{1,\,obs}/K_a^{1,\,int}$

Scheme 1.

isotope effect on this chemical shift (*15*), and the short H bond observed in crystals (*7*, *16*). The ^1H chemical shift in PA^- was confirmed, and the isotope fractionation factor of 0.56 (Table 1) is also consistent with an LBHB. The substituents at the 4-position on the benzene ring (Scheme 1), which is *para* to one of the carboxylic acid groups and *meta* to the other, differentially affect the acidities of the two otherwise identical carboxylic acid groups. This allows investigation of the H-bond strength over a range of ΔpK_a that includes $\Delta pK_a = 0$. Stabilization of the monoanions of substituted PAs by this H bond renders their first deprotonation more favorable, thereby decreasing their pK_a values (Scheme 1). The decrease of the experimentally observed pK_a^1 [$pK_a^{1,\,obs}$ (*17*)] from its "intrinsic" value [$pK_a^{1,\,int}$ (*18*)] provides a measure of the strength of the H bond (K_f^{HB}) (Table 2) (*19*).

A linear correlation between H-bond energy and ΔpK_a was found for the PA system (Fig. 2). No positive deviation was observed at $\Delta pK_a = 0$, in contrast to the prediction from the LBHB proposal. Analogous experiments have shown that there is also a linear correlation with no positive deviation near $\Delta pK_a = 0$ for the intramolecular H bond in a series of substituted salicylate monoanions (*20*). This correlation spans a wider range of ΔpK_a (~5 units in DMSO), although it does not include a fully symmetrical species with $\Delta pK_a = 0$.

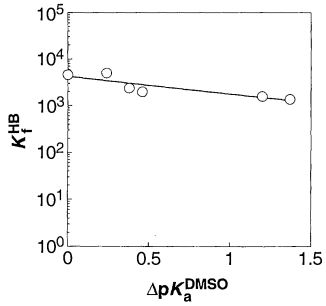
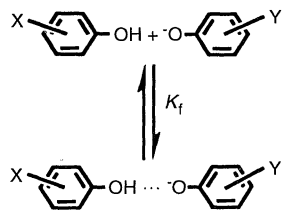


Fig. 2. Formation constants of the intramolecular H bonds as a function of ΔpK_a^{DMSO} for a series of substituted phthalate monoanions in DMSO [$\beta = 0.37$ with the ΔpK_a^{DMSO} scale, or $\beta = 0.9$ with the ΔpK_a^{water} scale (not shown)]. Data are from Table 2.

Studies of the H bonds in phthalate, maleate, and succinate monoanions suggested that single-minimum H bonds may only exist in solvents that are more nonpolar than is DMSO (*10*). Further, because covalent interactions have more stringent geometrical requirements than do electrostatic interactions, the potential formation of a very strong LBHB in PA^- may also be prevented by unfavorable geometrical fixation of the intramolecular H bonds.

We addressed these potential limitations (*21*) by using the more nonpolar solvent tetrahydrofuran (THF) and by investigating an intermolecular H bond. Thus, the donor and acceptor could adopt a particular H-bonding geometry if it were required for the formation of a very stable LBHB. Two series of phenols (X-ΦOHs) and phenolates were investigated. The proton chemical shifts of ~17 ppm observed previously for complexes of several phenols with their conjugate bases in nonaqueous solvents (*22*) are consistent with the formation of LBHBs. Formation constants (K_f) of H-bonded complexes of X-ΦOHs with 3,4-dinitrophenolate or 4-nitrophenolate,



$Y = 4\text{-}NO_2$ or $3,4\text{-}(NO_2)_2$ (1)

were measured spectrophotometrically (*23*) (Table 3). For each series, there is a linear correlation between the strength of H bonds (*24*) and ΔpK_a, without any deviation at $\Delta pK_a = 0$ (Fig. 3), as was observed for the PAs.

These results provide no indication that H bonding is significantly strengthened when the donor and acceptor pK_a's are matched, in contrast to the LBHB proposal. Analogous conclusions were drawn from recent ab initio calculations (*25*). A linear relation between H-bond energy and ΔpK_a has also been observed in previous studies of H bonding in aqueous and organic solutions (*12*, *14*, *26*) and in the gas phase (*5*, *13*).

The observed energetic properties of H bonds follow predictions from a simple electrostatic model,

$$E^H = \frac{k}{\varepsilon r}(q_1 q_2) \qquad (2)$$

(*11*, *27*). According to this model, changes in H-bond energy (E^H) can be described by changes in Coulombic interactions between partial effective charges on the H-bond donor and acceptor (q_1 and q_2, respectively), with an interaction coefficient that is dependent on the dielectric constant of the media (ε) and the distance separating the partial charges (r). E^H increases as the donor becomes more acidic (or the acceptor becomes more basic), because of an increase in partial positive charge on the donor (q_1) [or an increase in partial negative charge on the acceptor (q_2)]. Thus, E^H is maximal when $\Delta pK_a = 0$ ($q_1 = q_2$; Figs. 2 and 3) even in the absence of special energetic contributions from covalent character in an LBHB (*28*). However, the increase is not dependent on the matching of the pK_a's of the donor and acceptor, contrary to the prediction from the LBHB hypothesis; rather, it depends solely on the magnitude of the change in ΔpK_a.

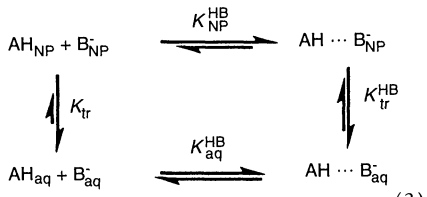
Another prediction from the electrostatic model is that E^H will increase with decreasing dielectric of the media (*29*). This prediction is supported by the observation that H-bond energy generally increases as

Table 2. Determination of H-bond energy in substituted phthalate monoanions from pK_a measurements in DMSO. Values of $pK_a^{1,\,obs}$, $pK_a^{1,\,int}$, and ΔpK_a were obtained as described in (*17*), (*18*), and (*45*), respectively. Values of log K_f^{HB} were calculated from log (K_a^{obs}/K_a^{int}) = ($pK_a^{int} - pK_a^{obs}$), derived from Scheme 1. Values of $-\Delta G_f^{HB}$ were calculated from $\Delta G_f^{HB} = -RT \ln K_f^{HB}$, where R is the gas constant (1.987 cal mol^{-1} K^{-1}) and T is temperature in kelvin (298 K).

Compound	$pK_a^{1,\,obs}$	$pK_a^{1,\,int}$	ΔpK_a	log K_f^{HB}	$-\Delta G_f^{HB}$ (kcal/mol)
PA	6.04 ± 0.13	9.70	0	3.66	5.0
4-Me-PA	6.10 ± 0.02	9.80	0.24	3.70	5.0
4-Cl-PA	5.43 ± 0.12	8.91	0.38	3.38	4.6
4-Br-PA	5.46 ± 0.05	8.76	0.46	3.30	4.5
4-OH-PA	6.21 ± 0.02	9.41	1.20	3.20	4.4
4-NH_2-PA	6.88 ± 0.08	9.92	1.37	3.04	4.1

the environment changes from water to organic solvents or to the gas phase (*5, 6, 12–14, 26*). The large H-bond energies in nonaqueous environments and their ability to undergo large changes because of electronic rearrangements in the course of a reaction may allow H bonds to provide substantial catalytic contributions (see below) (*20, 30, 31*).

Isolated charges are highly unstable in low-dielectric environments, so that the observed large formation energy of ionic H-bonded species in nonpolar media (K_{NP}^{HB}) may arise from the instability of charge separation in the nonpolar medium ($K_{tr} \gg 1$) rather than from a special character of these H bonds:



(3)

This inference is consistent with the observation that the formation of both symmetrical and asymmetrical ionic H-bonded species is highly favorable in the gas phase and that the formation of neutral H-bonded species is much less favorable (*5, 6, 13*). Further, if all other factors are equal, the H-bonded species itself is presumably more stable in water than in nonpolar media because the H bond is polar

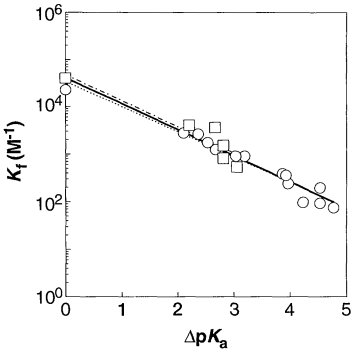


Fig. 3. Formation constants of the H-bonded complexes of substituted phenols with 3,4-dinitrophenolate (circles) or 4-nitrophenolate (squares) in THF as a function of ΔpK_a^{water}. The solid line represents the overall fit of the data for both series with $\beta = 0.65$; the lighter dashed lines represent the fit of individual series of data for H bonding with 3,4-dinitrophenolate ($\beta = 0.63$) or 4-nitrophenolate ($\beta = 0.65$). The ΔpK_a values in water were used because the values in THF have not been determined. These ΔpK_a^{water} values are expected to be proportional to those in THF (*38–40, 43*), but the range of ΔpK_a is expected to be >10 in THF (*42*). Data are from Table 3.

($K_{tr}^{HB} > 1$) (*32*). In addition, to lessen charge separation in nonpolar media, it is possible that the H-bonded proton may become more equally shared between the heteroatoms. Such a rearrangement could give rise to the unusual spectroscopic properties of H bonds in nonpolar media. Thus, the observed unusual spectroscopic properties of certain enzymatic H bonds (*4, 33*) may be a manifestation of the low dielectric of the enzymatic interior, and not a reflection of unusual energetic properties of LBHBs (*34*).

Our results provide no indication that LBHBs provide a special energetic contribution to enzymatic catalysis. In that case, how do H bonds contribute to enzymatic catalysis? Several possibilities are suggested by this and other model studies; the suggestions parallel several previous proposals (*30, 31*). For a given electronic rearrangement as a reaction proceeds from the ground state to the transition state, the increase in H-bond strength would be greater in a low-dielectric enzymatic active site than in aqueous solution. The different degree of strengthening of the H bond therefore allows greater transition-state stabilization for the enzymatic reaction (*20, 35*). A larger Brønsted slope (β) in nonaqueous media than in aqueous media, which represents a greater increase in H-bond strength in the nonpolar solvent as the donor-acceptor pair is varied, has been observed for a series of substituted salicylates [slopes of 0.70 in DMSO and 0.14 in water for log K^{HB} versus pK_a^{water} (*20*)]. Other examples of larger β values in nonaqueous media have been observed in this and previous studies (Figs. 2 and 3) (*5, 8, 14, 26, 36*). In addition to the strengthening of H bonding by electrostatic effects during an enzymatic reaction, the binding energy of an enzyme may be used to position substrates with respect to enzymatic catalytic groups, thus providing an entropic advantage for transition-state H bonding between the enzyme and the substrate. Further, H bonds may also be strengthened by geometrical changes in going from the ground state to the transition state, thereby preferentially stabilizing the transition state. Finally, enzymes may commonly use multiple interactions of moderate strength for transition-state stabilization, rather than relying on a single, very strong interaction such as an LBHB [see (*20, 30, 31, 37*) for more detailed discussions].

Table 3. Formation constants (K_f) of H-bonded complexes of substituted phenols (X-ΦOHs) with 3,4-dinitrophenolate [3,4-$(NO_2)_2$-ΦO$^-$] or 4-nitrophenolate (4-NO_2-ΦO$^-$) at 25°C. Tetrabutylammonium ion was used as the counterion. Values of ΔpK_a^{water} were obtained by subtracting pK_a^{water} (donor) from pK_a^{water} (acceptor) (*46*). Values of K_f were determined as described in (*23*). Values of $-\Delta G_f^{HB}$ were calculated from $\Delta G_f = -RT \ln K_f$, where R is the gas constant (1.987 cal mol^{-1} K^{-1}) and T is temperature in kelvin (298 K).

H-bond donor	H-bond acceptor	ΔpK_a^{water}	K_f (10^3 M^{-1})	$-\Delta G_f$ (kcal/mol)
3,4-$(NO_2)_2$-ΦOH	3,4-$(NO_2)_2$-ΦO$^-$	0	24	6.0
3,4,5-Cl_3-ΦOH		2.10	2.8	4.7
4-Cl-3-NO_2-ΦOH		2.36	2.6	4.7
4-CN-ΦOH		2.53	1.7	4.4
3,5-Cl_2-ΦOH		2.67	1.3	4.2
3,4-Cl_2-ΦOH		3.03	0.91	4.0
3-CN-ΦOH		3.19	0.91	4.0
3,4-F_2-ΦOH		3.86	0.38	3.5
4-Br-ΦOH		3.92	0.36	3.5
4-Cl-ΦOH		3.96	0.24	3.2
3-OMe-ΦOH		4.23	0.20	3.1
4-F-ΦOH		4.53	0.10	2.7
ΦOH		4.53	0.091	2.7
3,5-Me_2-ΦOH		4.77	0.077	2.6
4-NO_2-ΦOH	4-NO_2-ΦO$^-$	0	40	6.3
4-Br-ΦOH		2.20	4.0	4.9
4-Cl-ΦOH		2.66	3.6	4.8
4-F-ΦOH		2.81	1.5	4.3
ΦOH		2.81	0.83	4.0
3,5-Me_2-ΦOH		3.05	0.53	3.7

REFERENCES AND NOTES

1. LBHB is used herein to denote H bonds that exhibit anomalous spectroscopic properties and have been observed or proposed to be very strong (>10 kcal/mol). For simplicity, it is meant to include all the terms frequently used for this "special" type of H bond, including low-barrier H bonds, single-well or symmetrical H bonds, and short, strong H bonds. "LBHB proposal" refers to the proposed energetic contribution of LBHB at $\Delta pK_a = 0$ to catalysis, not to the bond length or nature of the H-bond potential.
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17. pK_a's in DMSO were measured by the overlapping indicator method (*38–40*). Indicators used were 2,4-dinitrophenol (pK_a = 5.12), 2,6-di-*tert*-butyl-4-nitrophenol (pK_a = 7.30), and 9-carboxymethylfluorene (pK_a = 10.35), which were synthesized as described (*38, 39*). Each pK_a in Table 2 was measured with two different indicators whose pK_a's bracketed that of the compound of interest. The pK_a's below 6 were also confirmed by measurement of self-dissociation of the acid (*40*). Values are reported as mean ± 2 SD.
18. The intrinsic pK_a (p$K_a^{1,\,int}$) represents the pK_a of the carboxylic acid expected in the absence of H bonding. For PA, this value was obtained in two ways: from the pK_a of the monomethyl ester of PA (9.60 ± 0.01) and from the pK_a^1 of terephthalic acid (9.80 ± 0.06), in which the two carboxylic acid groups are *para* to one another instead of *ortho*. (The pK_a of PA monomethyl ester has been statistically corrected for comparison with the two ionizable carboxylic acid groups in the phthalic acids.) In both cases, the neighboring H-bond donor is removed and replaced by a substituent that is expected to have a similar effect on the acidity of the carboxylic acid but cannot form an H bond with the carboxylate. For substituted PAs (X-PAs), the first deprotonation can be from the carboxylic acid either *para* or *meta* to the substituent. The p$K_a^{1,\,int}$ value for each of the X-PA carboxylic acid groups was calculated from

$$pK_a^{1,\,int}(\text{X-PA}) = pK_a^{1,\,int}(\text{PA}) - \rho\sigma_{\text{X, meta or para}} \quad (4)$$

where σ is the Hammett constant describing the electron-withdrawing ability of benzyl substituents, ρ is the slope of the linear dependence of pK_a on the σ values of the substituents for a homologous series of compounds, and "X, *meta* or *para*" refers to the substituent in either *meta* or *para* position (the lower of the two calculated values reflects the group that will be deprotonated first). Values of σ are from O. Exner, *Correlation Analysis of Chemical Data* (Plenum, New York, 1988), pp. 439–540. A ρ value of 2.4 in DMSO (*40*) was used.
19. The increase in the second pK_a of PA and related compounds relative to the first pK_a has been considered to be evidence for the strength of the H bond in PA^-. However, formation of the H bond both lowers pK_a^1 and raises pK_a^2. In addition, electrostatic repulsion in the dianion renders the second deprotonation unfavorable (Scheme 1). Compounds with negative charges or lone pairs positioned close to one another are expected to have greater proton affinity (that is, higher pK_a's) than do compounds that lack such destabilization of the deprotonated species. The increase of pK_a^2 relative to pK_a^1 therefore overestimates the inherent strength of the H bond and was not used. This expectation holds whether or not the donor and acceptor pK_a's are matched.
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21. An additional limitation of the intramolecular H-bonding system (PA series) is that the pK_a's of both donor and acceptor are changed by the substituent as ΔpK_a is varied. Some deviation from a simple linear relation might therefore be anticipated. Such deviations are expected to be small because the pK_a's vary over a limited range, and because the value of β for H-bond strength versus pK_a has a small dependence on the pK_a of the donor or acceptor (*11, 12*) (Fig. 3). This limitation is eliminated in the phenol-phenolate series (Table 3 and Fig. 3), as the pK_a's of the donor and acceptor are varied independently in the intermolecular system.
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23. Formation constants of the H-bonded complexes (K_f, Eq. 1) were measured from the spectral shift of free nitro-substituted phenolates to lower wavelengths upon H bonding (λ_{max} = 420 nm → λ_{max} = 388 nm) [I. M. Kolthoff and M. K. Chantooni Jr., *J. Am. Chem. Soc.* **93**, 3843 (1971)]. The K_f values were obtained by fitting the absorbance decrease at 430 nm to a binding curve,

$$\frac{A_{430}}{C_{\Phi O^-}} = \varepsilon_{430}^{\Phi O^-} b\left(\frac{1/K_f}{C_{\Phi OH} + 1/K_f}\right) + \varepsilon_{430}^{cplx} b\left(\frac{C_{\Phi OH}}{C_{\Phi OH} + 1/K_f}\right) \quad (5)$$

where $A_{430}/C_{\Phi O^-}$ is the normalized total absorbance of the phenolate, expressed as the sum of contributions from the fraction in the free form, $\varepsilon_{430}^{\Phi O^-} b[(1/K_f)/(C_{\Phi OH} + 1/K_f)]$, and the fraction in the H-bonded complex, $\varepsilon_{430}^{cplx} b[C_{\Phi OH}/(C_{\Phi OH} + 1/K_f)]$; b is the pathlength (1 cm); $C_{\Phi OH}$ and $C_{\Phi O^-}$ are the total concentrations of the phenol and phenolate, respectively; and $\varepsilon_{430}^{\Phi O^-}$ and ε_{430}^{cplx} are the extinction coefficients of the phenolate in free and complexed form, respectively. Titration of each phenolate with increasing amounts of the phenol revealed a single isosbestic point at ~405 nm, which confirmed the transition of the phenolate between two species, free phenolate and its H-bonded complex. The phenol concentration was varied over a range of 0.2 to 5 K_d (the dissociation constant of the phenol-phenolate complex; $K_d = 1/K_f$) or greater. The values of ε_{430}^{cplx} obtained for each of the heteroconjugates and for the homoconjugate were small, and the overall spectra of the complexes were similar. For the homoconjugates, the spectra (from 300 to 500 nm) of mixtures of the phenolate, its conjugate acid, and the complex could readily be deconvoluted to give the concentration of each species with the use of the experimentally determined spectra for the individual species. The value of K_f obtained from the deconvoluted concentrations (at each of several initial phenol-phenolate concentrations) was similar to the values obtained from the above fit to the absorbance change at 430 nm. On the basis of a conservative estimate from the range of K_f values that fit the data reasonably, there is an uncertainty of 0.3 (log 2) in the reported log K_f values; this does not affect the conclusion concerning whether there is a deviation at ΔpK_a = 0, nor does it change the slopes in Fig. 3 significantly (*41*).
24. The ΔG_f values represent free energy of complexation, which includes processes other than H bonding. However, because we used a homologous series of compounds, effects from other factors involved in complexation are expected to be constant and not to perturb the dependence on ΔpK_a (that is, the slope). Analogous considerations hold for the intramolecular H bonds in substituted PAs.
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27. We do not mean to imply that H bonds have no covalent character. Indeed, the unusual spectroscopic behavior cited in the text is most simply accounted for by some degree of covalent character of certain H bonds (*7, 8*). Rather, our discussion deals solely with the energetic behavior of H bonds. The results suggest that the energetics can be understood from simple electrostatic considerations, and that covalent bonding character does not lead to unusual energetic behavior of these H bonds. Further, the near additivity of the energy of the two H bonds in 2,6-dihydroxybenzoate in DMSO suggests that these H bonds are not predominantly covalent in nature (*37*).
28. E^H cannot be increased infinitely by increasing the acidity of the donor (or increasing the basicity of the acceptor), because the proton is transferred when ΔpK_a = 0 is traversed. This is not accounted for in Eq. 2.
29. The use of the bulk dielectric constant ε in Eq. 2 is a simplification included to illustrate the effect of solvent properties on H bonding. Local solvation effects are important determinants of the stability of H-bonded species [for examples, see C. Beeson and T. A. Dix, *J. Am. Chem. Soc.* **115**, 10275 (1993); T. M. Krygowsk and W. R. Fawcett, *ibid.* **97**, 2143 (1975); A. Allerhand and P. R. Schleyer, *ibid.* **85**, 371 (1963); E. B. Nadler and Z. Rappoport, *ibid.* **111**, 213 (1989)].
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35. For an enzyme to take advantage of the greater increase of H-bond strength in nonaqueous environments, binding interactions away from the site of electronic rearrangement are presumably required to localize the H-bonding groups in the low-dielectric active site; this situation is not intrinsically favorable (*20, 32*).
36. The symmetrical H bond in the citraconate monoanion has been estimated to be ~4.4 kcal/mol stronger than the pK_a-mismatched diacid and monoamide in DMSO; this is larger than the difference of ~1 kcal/mol observed in methanol [B. Schwartz and D. G. Drueckhammer, *J. Am. Chem. Soc.* **117**, 11902 (1995)]. These differences may result from electrostatic and solvation effects that give a greater dependence of H-bond energy on ΔpK_a in DMSO than in methanol. Brønsted slopes for H bonding of 0.05 to 0.17 and 0.7 to 0.9 have been observed in methanol and DMSO, respectively (*11, 20*) (Fig. 2), using the same scale of pK_a^{water} to compare the results in the different solvents (*42*).
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41. Analysis of these spectra can be complicated by proton transfer between added phenol and the nitrophenolate, which depletes the total amount of nitrophenolate available for H bonding and causes spectral shift of the nitrophenolate beyond that from H bonding. To avoid this problem, we used phenols whose pK_a values were considerably higher than those of the nitrophenolate, the proton acceptor. However, at high phenol concentrations, phenol can self-associate and lose a proton to give the phenol•phenolate complex, thereby allowing protonation of the nitrophenolate. For this reason, K_f values were only obtained in the region $\Delta pK_a^{water} > 2$, where the proton transfers occur at much higher phenol concentrations than are required for complex formation with the nitrophenolate. Control experiments with sterically hindered phenols (2,6-di-*tert*-butylphenol and 2,6-di-*tert*-butyl-4-nitrophenol) did not produce the spectral shift described in (*23*), even at concentrations up to 1 M. This finding ruled out complications from general solvent effects by addition of phenols to the phenolate solution and suggested that H bonding is responsible for complexation. Addition of increasing concentrations of a nonconjugate phenol to a solution containing a preformed homoconjugate nitrophenol-nitrophenolate complex leads to loss of the homoconjugate complex and formation of the heteroconjugate complex, as judged by modest spectral shifts. The concentration dependence of these changes was consistent with the relative stability of the H-bonded species obtained from the direct measurements described in (*23*).
42. The pK_a scales are substantially expanded in low-dielectric solvents. For example, for benzoic acids and phenols, ΔpK_a's of 1 in water correspond to ΔpK_a's of ~2.4 in DMSO (*40*). For THF, the increase of the ΔpK_a scales is expected to be somewhat greater (*43*).
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44. Isotope fractionation factors (the D/H isotopic ratio for the H-bonded proton relative to the ratio for the water protons) were measured by ^1H nuclear magnetic resonance (NMR) spectroscopy. A small amount (~0.2 to 0.3%) of a D_2O/H_2O mixture was added to 0.2 M PA^- solutions in DMSO. The deuterons in the added isotope-containing water were allowed to exchange and reach equilibrium with the COOH protons. The slowly exchanging water and PA^- proton peaks were integrated and normalized to that of the nonexchangeable benzylic protons. The ratio of the two normalized peak areas, in comparison with that in a control sample where the same amount of H_2O was added, yielded the fractionation factors [M. Saunders, S. Saunders, C. Johnson, *J. Am. Chem. Soc.* **106**, 3098 (1984)]. To improve the accuracy of data, we performed multiple measurements using different D_2O/H_2O ratios (1/9 → 9/1). The small amount of water added did not affect the observed downfield chemical shift of the H-bonded proton. Values are reported as mean ± 2 SD.
45. ΔpK_a values are defined as the difference between the intrinsic pK_a's (pK_a^{int}) (*18*) of the H-bond donor and acceptor. For X-PAs, this reduces to $\Delta pK_a = \rho(|\sigma_{X,para} - \sigma_{X,meta}|)$.
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47. We thank J. Brauman, J. Klinman, and J. Kirsch for discussions and helpful suggestions, the Bordwell group for technical help, and the Herschlag lab for comments on the manuscript. This work is supported by grants from the Lucille P. Markey Charitable Trust and the Chicago Community Trust to D.H. D.H. is a Lucille P. Markey Scholar and a Searle Scholar.

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Age-Dependent Diarrhea Induced by a Rotaviral Nonstructural Glycoprotein

Judith M. Ball, Peng Tian,* Carl Q.-Y. Zeng, Andrew P. Morris, Mary K. Estes†

The rotavirus nonstructural glycoprotein NSP4 is an intracellular receptor that mediates the acquisition of a transient membrane envelope as subviral particles bud into the endoplasmic reticulum. NSP4 also causes an increase in intracellular calcium in insect cells. Purified NSP4 or a peptide corresponding to NSP4 residues 114 to 135 induced diarrhea in young (6 to 10 days old) CD1 mice. This disease response was age-dependent, dose-dependent, and specific. Electrophysiologic data from intestinal mucosa showed that the NSP4 114-135 peptide potentiates chloride secretion by a calcium-dependent signaling pathway. Diarrhea is induced when NSP4, acting as a viral enterotoxin, triggers a signal transduction pathway.

Rotaviruses are the leading cause of severe, life-threatening viral gastroenteritis in infants and animals (*1*) and are associated with sporadic outbreaks of diarrhea in elderly (*2*) and immunocompromised patients (*3*). These viruses have a limited tissue tropism, with infection primarily being restricted to cells of the small intestine (*4*). Moreover, the outcome of infection is age-related; although rotaviruses may infect individuals and animals of all ages, symptomatic infection (that is, diarrhea) generally occurs in the young (6 months to 2 years in children and up to 14 days in mice).

Despite the prevalence of rotavirus infections and extensive studies in animal models, rotavirus pathogenesis remains poorly understood. Proposed pathophysiologic mechanisms by which rotaviruses induce diarrhea after virus replication include malabsorption secondary to the destruction of enterocytes (*5*), alterations in transepithelial fluid balance (*6*), and local villus ischemia leading to vascular damage and diarrhea (*7*). These mechanisms do not explain cases of rotavirus-induced diarrhea observed before, or in the absence of, histopathologic changes (*4*, *8*).

While making an antiserum to a nonstructural glycoprotein, NSP4, we made the fortuitous discovery that intraperitoneal (ip) delivery of purified NSP4 induces diarrhea in a mouse model (Fig. 1). Whether administration was ip or intraileal (il), diarrhea was observed within 1 to 4 hours after inoculation. It typically continued for up to 8 hours, but occasionally persisted for 24 hours (*9*). Purified NSP4 (0.1 to 5 nmol) was administered by the ip route to CD1 mouse pups 6 to 7 and 8 to 9 days old. No diarrhea was induced with 0.1 nmol of protein in the 8- to 9-day-old mice, whereas 60% of the 6- to 7-day-old pups had diarrhea. Intraperitoneal administration of 1 nmol of NSP4 resulted in diarrhea in 100% of the 6- to 7-day-old mice, and in 60% of the older animals. Intraileal delivery of 0.5 nmol of protein induced diarrhea in 100% of the young (8 to 9 days) mice, whereas no diarrhea was observed in the 17- to 18-day-old pups. Thus, the response to NSP4 was age- and dose-dependent in CD1 pups. In addition, the induction of diarrhea by NSP4 was specific, as administration of the same concentration of purified rotavirus VP6 or the same volume of buffer had no effect (Fig. 1). Intramuscular (im) inoculation of 1 nmol of purified NSP4 produced no ill effects (*10*).

We next tested the effect of a synthetic peptide corresponding to NSP4 residues 114 to 135 (NSP4 114-135) delivered by the ip or il route to mice of different ages (Fig. 2) (*9*, *11*). Diarrhea was observed in the 6- to 7-day-old mice within 1 to 3 hours after inoculation [60% (ip), 71% (il)], whereas diarrhea was not seen in animals older than 11 days, even when a two- or fourfold greater dose of peptide was administered ip (Fig. 2). Intraileal delivery of the peptide to pups 11 to 12 or 17 to 18 days old caused diarrhea in 25 or 0% of the animals, respectively. These data indicate an increased sensitivity to the peptide when delivered directly into the lumen of the intestine, and reveal an age-dependent disease response to the NSP4 114-135 peptide that is similar to that seen in natural rotavirus infections or after inoculation of purified NSP4. Regardless of the dose or the route of administration of the peptide, the kinetics of diarrhea induction were similar to those observed with purified NSP4. However, as compared with NSP4, the effective dose of NSP4 114-135 peptide was considerably

J. M. Ball, P. Tian, C. Q.-Y. Zeng, M. K. Estes, Division of Molecular Virology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA.
A. P. Morris, Department of Integrative Biology, University of Texas Health Science Center, Houston, TX 77030, USA.

*Present address: Howard Hughes Medical Institute and Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, USA.
†To whom correspondence should be addressed.