Analysis of Energetic Contributions

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Overview

Enzymes typically form hydrogen bonds with substrate moieties that undergo electronic rearrangement in the course of reaction. This article reviews results that suggest that these hydrogen bonds can be strengthened in the transition state to a greater extent within the context of the enzymatic active site than in aqueous solution. Comparisons in model systems demonstrate this differential strengthening effect and suggest that it may provide substantial rate enhancements for enzymatic reactions relative to solution reactions.\textsuperscript{1-3} Despite their likely importance, quantitative dissection of the role of these "reaction center" hydrogen bonds in enzymatic catalysis is blurred because the energetic contributions of these hydrogen bonds are intertwined with those of hydrogen bonds and other interactions that fold the enzyme and position the bound substrate. This interconnection between binding interactions and rate enhancement is a fundamental property of enzymatic catalysis.\textsuperscript{4}

The Hydrogen Bond: General Energetic Considerations

When a hydrogen attached to a heteroatom is near van der Waals' contact with another heteroatom, a hydrogen bond is typically inferred. Although energy cannot be read from structure, the preponderance of such contacts, with preferred distances and angles, suggests an energetic preference for such interactions.\textsuperscript{5-11} From an energetic standpoint, when a

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complex is formed in which the predominant identifiable interaction is that between a heteroatom-bonded hydrogen atom of one molecule and a heteroatom of a second species, such as that between ROH and F⁻ in the gas phase [Eq. (1); \( \Delta H^\circ = -32 \text{ kcal/mol} \)], we attribute the driving force for complex formation to the hydrogen bond:

\[
\text{EtOH} + F^- \xrightleftharpoons[\Delta H^\circ = -32 \text{ kcal/mol}]{K_{\text{HB}}} \text{EtOH} \cdot F^- 
\] (1)

However, the above picture of the hydrogen bond is deceptively simple. Whereas the equilibrium formation of the hydrogen-bonded EtOH \cdot F⁻ complex [Eq. (1)] would appear to represent one of the most straightforward cases in which hydrogen bonding is the clear driving force for complex formation, F⁻ also has large association energies for the formation of complexes that would not typically be described as hydrogen bonding. For example, the substantial enthalpy value of \( \Delta H^\circ = -30 \text{ kcal/mol} \) for forming the CF₃—CHF₂ · F⁻ complex [Eq. (2)] suggests that F⁻ interacts strongly with dipolar species in the gas phase, presumably because of its high charge density and the absence of electrostatic screening from solvent. Such observations caution against the view that all of the enthalpy change from formation of the EtOH · F⁻ complex [Eq. (1)] should necessarily be considered a simple measure of “hydrogen bond strength.”

\[
\text{CF₃}—\text{CHF₂} + F^- \xrightleftharpoons[\Delta H^\circ = -30 \text{ kcal/mol}]{K'} \text{CF₃}—\text{CHF₂} \cdot F^- 
\] (2)

To analyze the energetics of molecular processes quantitatively, such as the equilibria of Eqs. (1) and (2), an overall process is often broken down into energetic contributions from components that can be considered as “transferable properties,” but this appears to be problematic for hydrogen bonding. A property is “transferable” if the same energy, to a reasonable approximation, is obtained for that component regardless of surroundings. Strictly speaking, a change in free energy (or enthalpy) is always a property of an entire system, not an isolated component of the system. In practice, an overall process can often be dissected using components that are, to a reasonable approximation, transferable; this facilitates understand-

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ing of the complex system under investigation.\textsuperscript{14,15} For example, additivity holds to a first level of approximation for covalent bonds and allows reasonable calculations of enthalpies of formation for many compounds. However, complexation via hydrogen bonding appears to be highly sensitive to molecular context and solvent (see later). It appears that hydrogen bonds cannot be treated as simple transferable properties.

Analyses of hydrogen bonds in this article rely on discrete comparisons. This is both because of the difficulty in assigning "absolute" or transferable energies to hydrogen bonds, as described earlier, and because we are, in fact, interested in the relative energetics of hydrogen bonds. We would like to know how the energy of a hydrogen bond changes in a transition state relative to a ground state, and further, we would like to compare this change in energy on an enzyme to the change in solution. We suggest that much of the confusion about hydrogen bond and other energetic features of biological systems has arisen because comparisons are often made implicitly, rather than explicitly. Conversely, explicit comparisons, such as those described later, represent powerful analytical tools to probe aspects of hydrogen bonds that may be important in enzymatic catalysis.

Can Strengthened Transition-State Hydrogen Bonding Contribute to Enzymatic Catalysis?\textsuperscript{16}

The Catalytic Problem

Charge rearrangement typically occurs in the course of a reaction. This is shown in Scheme 1 using the triosephosphate isomerase (TIM) reaction as an example. As the reaction proceeds from the ground state to the transition state, negative charge develops on the carbonyl oxygen of the substrate. Hydrogen bonds from His-95 and Lys-12 of TIM to the carbonyl


\textsuperscript{16} Phrases such as "strengthening of hydrogen bonds" and "increase in hydrogen bond strength" are used in the text to refer to increases in the equilibrium constant for the formation of hydrogen-bonded complexes caused by changing local electrostatics [e.g., the charge buildup on the substrate carbonyl oxygen in the transition state for the TIM reaction (Scheme 1)]. Based on simple electrostatic considerations, an increase in "hydrogen bond strength" is expected when there is an increase in electron density on the hydrogen bond acceptor and/or a decrease in electron density on the hydrogen bond donor, with all other factors being equivalent. It is hoped that the suggestion of comparison in these phrases provides a sufficient reminder that equilibria for hydrogen bond formation need to be compared, as hydrogen bond energies cannot be defined as simple transferable properties.
oxygen\textsuperscript{17} (Scheme 1, bottom) become stronger as a result of this charge buildup, as depicted by the darker dots in the transition state. However, in the solution reaction, the hydrogen bonds from water to the substrate also become stronger in the transition state (Scheme 1, top reaction). Enzymes, of course, need to catalyze reactions relative to reactions in aqueous solution. Thus, the strengthening of hydrogen bonds at the active site must be greater than the strengthening of the corresponding hydrogen bonds to water.

It is well established that enzymes can achieve some of their catalysis by positioning active site moieties with respect to bound substrate\textsuperscript{4,18} (see also later). For example, in the active site of TIM, hydrogen bond donors and acceptors and the general acid and base are positioned in the active site with respect to bound substrate so that the entropic cost of arranging interactions that stabilize the transition state is overcome. Here we address a conceptually distinct question about the potential catalytic role of hydrogen bonds: After accounting for effects from positioning, are there differences between the active site and aqueous solution that allow a larger increase in hydrogen bond strength on the enzyme than in aqueous solution accom-

panying the electronic rearrangements in going from the ground state to the transition state?

**Potential Solutions to the Catalytic Problem**

The following sections describe model studies that suggest that this strengthening of hydrogen bonds to enzymatic groups in the course of a reaction can be greater than the corresponding strengthening of hydrogen bonds to water. The general approach is to probe hydrogen-bonding equilibria with homologous series of compounds. The change in pK_a of these compounds as substituents is varied and is used as a mimic of the change in charge distribution of the substrate functional groups in going from the ground state to the transition state.

**Stronger Hydrogen Bond Donors and Acceptors at Enzymatic Active Sites Can Provide Greater Strengthening of Hydrogen Bonds Accompanying Charge Rearrangement.** Remarkably, simple complexes with only a single hydrogen bond, such as that between a phenolate and a protonated amine [Eq. (3)], can be observed in aqueous solution despite the competition for hydrogen bonding by 55 M water.\(^\text{19}\)

\[
\begin{align*}
\text{Ph}^{-} \cdot \text{HOH} + \text{H}_2\text{O} \cdot ^{+}\text{HNR}_3 & \rightleftharpoons \\
\text{Ph}^{-} \cdot ^{+}\text{HNR}_3 + \text{H}_2\text{O} \cdot \text{HOH (aq)}
\end{align*}
\]  

(3)

While it is intuitive that the increased negative charge density on the phenolate ion relative to the oxygen of water would increase the strength of the electrostatic interaction with a protonated amine donor,

\[
\left[ \text{Ph}^{-} \cdot ^{+}\text{HNR}_3 \text{vs H}_2\text{O} \cdot ^{+}\text{HNR}_3 \text{in Eq. (3)} \right],
\]

the interaction with a water donor would also be stronger:

\[
\text{Ph}^{-} \cdot \text{HOH vs H}_2\text{O} \cdot \text{HOH in Eq. (3)}.
\]

The ability to form the \(\text{Ph}^{-} \cdot ^{+}\text{HNR}_3\) complex indicates that the increase in hydrogen bond strength is dependent on both the donor and the acceptor, i.e., the increase in hydrogen bond strength upon changing

the acceptor from H$_2$O to RO$^-$ is larger with the stronger hydrogen bond donor, $^+$HNR$_3$, than it is with HOH.$^{19,20}$

How does this relate to hydrogen bonding in enzymatic catalysis? Consider the reaction catalyzed by TIM (Scheme 1) in which the carbonyl oxygen accumulates negative charge in going from the ground state (C=O) to the transition state (C=O$^{6-}$). For a given hydrogen bond donor, the hydrogen bond strengthens in the transition state relative to the ground state because of this increased charge density on the acceptor. What happens with different hydrogen bond donors, i.e., water for the solution reaction vs Lys-12$^+$ for the enzymatic reaction (Fig. 1, top and bottom, respectively)? The positively charged Lys-12$^+$ is a stronger hydrogen bond donor than water. This causes a larger strengthening of the hydrogen bond to the carbonyl oxygen in going from the ground state to the transition state on TIM (Fig. 1, $\Delta\Delta G_{\text{EHB}}^\text{ON}$) than in aqueous solution (Fig. 1, $\Delta\Delta G_{\text{soln}}^\text{ON}$), with all other factors being equal.

**What is the Basis for this Effect?** The larger increase in hydrogen bond strength with a stronger donor is predicted from a simple Coulombic model for electrostatic interactions between hydrogen bond donors and acceptors.$^{21}$ Equations (5a) and (5b) represent this Coulombic model for

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$^{19}$ The situation is analogous when the hydrogen bond donors and acceptors are switched in the descriptions throughout the text. This can be seen from the symmetry in Eq. (5) and the Hine equation [Eq. (8)] in the Appendix.

$^{20}$ It has been suggested that covalent character in hydrogen bonds between donors and acceptors with matched $pK_a$ values could result in large hydrogen bond energies in nonaqueous environments, thereby making a substantial contribution to enzymatic catalysis (e.g., Refs. 22–25). Indeed, it was these hypotheses that stimulated the experiments carried out in our laboratory.$^{1,3}$ The results and analyses suggest that hydrogen bonds may indeed contribute more to enzymatic catalysis than previously thought.$^{1,3}$ as suggested by these proposals. Nevertheless, data obtained thus far provide no indication of a large special energetic contribution arising from covalent character of hydrogen bonds (see also Refs. 2, 3, 26–31), and the energetic effects observed in model studies can be accounted for by simple electrostatic effects. Thus, a simple electrostatic model of hydrogen bonding is used in the text. In addition, the simplicity of this model facilitates an intuitive understanding of many of the factors that affect the energetics of hydrogen bonding. Nevertheless, structural and spectroscopic data strongly suggest that covalent interactions are also involved in certain hydrogen bonds in model and enzymatic systems, including TIM (Refs. 26–40; see also Refs. 5–7 and references therein). The nature of hydrogen bonds in enzymatic active sites and the physical properties of these hydrogen bonds that are important for their energetic behavior remain important areas for future investigation.


FIG. 1. The potential rate enhancement that can be obtained by a stronger hydrogen bond donor at the enzyme active site than water using the TIM reaction as an example.

the equilibria of Eqs. (4a) and (4b), respectively. In Eqs. (5a) and (5b), $q^+$
is the partial positive charge on the donor proton, $q^-$ and $q^-$
are the partial negative charges on the acceptor atoms in Eqs. (4a) and (4b), respectively;
the constant "C" describes the interaction between the donor and the

acceptor and depends on the distance between the partial charges (r) and the effective dielectric ($\varepsilon_{\text{eff}}$) of the media [Eq. (5c), see next section for a description of $\varepsilon_{\text{eff}}$; see Refs. 41–43 for a description of Coulomb's law]. Equation (5) indicates that the electrostatic interaction becomes stronger as the charge density on the donor or acceptor increases, i.e., if $q_i$ is more negative than $q_i^-$, then the value of $\Delta H_2^{HB}$ will be more negative than $\Delta H_1^{HB}$; this is represented by a negative $\Delta \Delta H^{HB}$ in Eq. (6).

Now consider the TIM reaction in solution and on the enzyme (Fig. 1). The more negative value of $q^-_2$ relative to $q^-_1$ is analogous to the increased negative charge density for the substrate carbonyl oxygen in the transition state relative to the ground state (Fig. 1; C=O$^-$ vs C=O). The hydrogen bond donor to the carbonyl oxygen on the enzyme (Lys-12$^+$) is stronger than water. This corresponds to an increase in $q^+$ (i.e., $q_{\text{sys}}>q_{\text{H-OH}}$) and results in a more negative value of $\Delta \Delta H^{HB}$ on the enzyme than in solution [Eqs. (7a) and (7b); $\Delta \Delta H^{HB}_{\text{soln}}$ is more negative than $\Delta \Delta H^{HB}_{\text{enzyme}}$]. Thus, the enzymatic hydrogen bond contributes more toward

$$\Delta H^{HB}_1 = C \times q^+ q^-_1$$

$$\Delta H^{HB}_2 = C \times q^+ q^-_2$$

$$C \propto \frac{1}{\varepsilon_{\text{eff}} T}$$

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35 T. K. Harris, C. Abeygunawardana, and A. S. Mildvan, Biochemistry 36, 14661 (1997).
preferentially stabilizing the transition state than the hydrogen bond in solution.

\[
\Delta\Delta H_{\text{HB}} = \Delta H_{2\text{HB}}^\text{H} - \Delta H_{1\text{HB}}^\text{H} = C \times q^+ (q^-_2 - q^-_1) = C \times q^+ \Delta q^- \quad (6)
\]

\[
\Delta\Delta H_{\text{HB}}^\text{soln} = C \times q^+_{\text{H}_2\text{O}} \Delta q^-
\]

\[
\Delta\Delta H_{\text{HB}}^\text{soln} = C \times q^+_{\text{H}_2\text{O}} \Delta q^-
\]

**Quantitative Estimates for the Catalytic Contribution from This Effect.** What is the magnitude of the possible catalytic effect stemming from the stronger hydrogen bond donor at the enzymatic active site relative to water? This can be estimated using the physical organic approach of linear free energy relationships. A brief introduction to linear free energy relationships is included because this approach is also important in the next section of this article.  

To construct a linear free energy relationship, the charge density on a hydrogen bond donor (or acceptor) is varied in a homologous series of compounds via electron-donating and electron-withdrawing substituents, as shown in Scheme 2 for substituted phenols. Changes in the \( pK_a \) of the \(-\text{OH}\) group provide a measure of the changes in the charge distribution at this position caused by the substituents. The equilibrium for hydrogen bond formation \( (K_{\text{HB}}) \) for the series of homologous compounds, such as phenolates ions, with a given hydrogen bond donor can be determined experimentally. Plots of the log of the equilibrium constant for hydrogen bond formation \( (\log K_{\text{HB}}) \) vs \( pK_a \) are typically linear and the slopes are referred to as Brønsted coefficients, \( \alpha \) values for hydrogen bond donors and \( \beta \) values for hydrogen bond acceptors (Figs. 2A and 2B, respectively).

Thus, the Brønsted slopes, \( \alpha \) and \( \beta \), describe the dependence of hydrogen bond formation on the charge distribution of the donor or acceptor (Figs. 2A and 2B, respectively) relative to the dependence of the protonation equilibrium. Small slopes of \( \alpha \) or \( \beta \approx 0.1-0.2 \) have been observed for such correlations in water and other protic solvents (Table 1). The small slopes are consistent with the idea that substituents have smaller effects on the largely electrostatic hydrogen bond \( (\log K_{\text{HB}}) \) than on covalent bond

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44 For more complete treatments of linear free energy relationships, see, for example, Refs. 45–48.


formation with a proton (pK$_a$). Note that an $\alpha$ or $\beta$ value of 1 would be obtained if hydrogen bond formation had the same dependence on substituents as protonation to give the phenol.

To quantitatively assess the possible catalytic effect stemming from the stronger hydrogen bond donor at the enzymatic active site relative to water, the Brønsted slope of a series of acceptors with one donor can be compared to the Brønsted slope for the same series of acceptors with a different donor. The different donors are meant to model the different hydrogen bond donors in the enzymatic and solution reactions (e.g., Lys-12' in TIM and water in solution). It has been observed that the value of $\beta$ increases when the linear-free energy relationship is obtained with a compound that is a stronger hydrogen bond donor (i.e., lower pK$_a$) (Fig. 2B). This is precisely what is predicted from Eqs. (7a) and (7b). Equations (7a) and (7b) describe this effect in terms of different changes in the predicted energy for hydrogen bond donors with different partial charges, whereas

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Fig. 2. Theoretical Brønsted plots of the equilibrium for H bonding ($K^{HB}$) between protonated amines and phenolate anions in water according to the Hine equation. Parameters used in the calculation are $\tau = 0.013$, $pK_a^{H_2O} = 16.04$, and $pK_a^{H_2O} = -1.26$.\(^{19,45}\) (A) Dependences of $\log K^{HB}$ on the $pK_a$ values of the hydrogen bond donor ($^+HNR_2-X$) described by the Brønsted slope $\alpha$. A steeper Brønsted slope is obtained with a stronger hydrogen bond acceptor, with $pK_a = 12$ (□), than with a weaker hydrogen bond of $pK_a = 4$ (○). (B) Dependencies of $\log K^{HB}$ on the $pK_a$ values of hydrogen bond acceptor

\[
\begin{align*}
\text{X} & \equiv \text{O}^- + ^+\text{HNR}_2-\text{X} \\
pK_a (^+\text{HNR}_2-\text{X}) & \equiv \alpha
\end{align*}
\]

described by the Brønsted slope $\beta$. The $\beta$ value is larger with a more acidic hydrogen bond donor with $pK_a = 4$ (○) than the $\beta$ value with a weaker donor with $pK_a = 12$ (□).

The increase in the Brønsted slope describes the same effect in terms of the observed equilibrium constant for hydrogen bond formation with hydrogen bond donors that have different $pK_a$ values.

The change in the value of $\beta$ with a stronger hydrogen bond donor can be estimated using the Hine equation, which quantitatively describes hydrogen bonding between solutes in water as a competition between solute · solute interactions and solute · solvent interactions, based on an electrostatic model for hydrogen bonding.\(^{19,45,52,53}\) The reader is referred

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to the Appendix for a detailed description of the Hine equation and the calculation of the rate enhancement from the stronger hydrogen bond donor, Lys-12$^+$ on the enzyme, relative to water. This analysis gives an estimated rate enhancement of 10-fold, assuming that the active site environment is the same as aqueous solution. This estimate presumably represents a conservative lower limit because the effect would be accentuated in an environment of low effective dielectric, as the enzymatic active site is thought to provide (see later). The next section describes that a low effective dielectric in an enzymatic active site can cause the increase in hydrogen bond strength in going from the ground state to the transition state to be larger than the corresponding increase in aqueous solution, whether or not the enzyme provides a stronger hydrogen bond donor than water.

**A Low Dielectric Environment Can Increase the Change in Hydrogen Bond Strength Accompanying Charge Rearrangement.** The electrostatic model for hydrogen bonding presented in the last section predicts that decreasing the “effective dielectric” of the media can also increase the strengthening of hydrogen bonds that accompanies changes in the charge distribution of donor or acceptor atoms [$\Delta \Delta H \propto 1/e_{\text{eff}}$; Eqs. (7) and (5c)]. The term “effective dielectric” is used to describe the effect of the particular
# Partial List of Observed Bronsted Slopes for Hydrogen Bonding in Aqueous and Organic Solvent

<table>
<thead>
<tr>
<th>Hydrogen bond</th>
<th>Solvent</th>
<th>Bronsted slope ( (\alpha \text{ or } \beta)^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( X^--O^- \cdot ^+HNR_3 )</td>
<td>Water</td>
<td>( \beta = 0.10^b )</td>
</tr>
<tr>
<td>( X^--O^- \cdot ^+HNR_2 - X )</td>
<td>Water</td>
<td>( \alpha = 0.15^b )</td>
</tr>
<tr>
<td>( X^--COO^- \cdot H )</td>
<td>Water</td>
<td>( \beta = 0.05^c )</td>
</tr>
<tr>
<td>( X^--COO^- \cdot COOH )</td>
<td>Dimethyl sulfoxide</td>
<td>( \beta = 0.73^c )</td>
</tr>
<tr>
<td>( H_3C-NH^+ \cdot N )</td>
<td>Acetonitrile</td>
<td>( \beta = 0.90^d )</td>
</tr>
<tr>
<td>( H_3CO-CH_2-OH \cdot X )</td>
<td>Acetonitrile</td>
<td>( \beta = 0.15^e,f )</td>
</tr>
<tr>
<td>( O^- \cdot HNBU )</td>
<td>Dimethyl acetamide</td>
<td>( \beta = 0.14^h )</td>
</tr>
<tr>
<td>( X^-OH \cdot O=\underset{\text{CH}_3}{\text{N}} )</td>
<td>1,1,1-Trichloroethane</td>
<td>( \alpha = 0.41^i )</td>
</tr>
<tr>
<td>( X^-ROH \cdot O=\underset{\text{CH}_3}{\text{N}} )</td>
<td>1,1,1-Trichloroethane</td>
<td>( \beta = 0.24^i )</td>
</tr>
<tr>
<td>( X^-OH \cdot O=\underset{\text{CH}_3}{\text{N}} )</td>
<td>1,1,1-Trichloroethane</td>
<td>( \beta = 0.40^i )</td>
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(continued)
<table>
<thead>
<tr>
<th>Hydrogen bond</th>
<th>Solvent</th>
<th>Bronsted slope $(\alpha \text{ or } \beta)^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="image" /></td>
<td>Tetrahydrofuran</td>
<td>$\alpha = 0.65^{d}$</td>
</tr>
<tr>
<td><img src="image2.png" alt="image" /></td>
<td>Carbon tetrachloride</td>
<td>$\alpha = 1.2^{i,f}$</td>
</tr>
<tr>
<td><img src="image3.png" alt="image" /></td>
<td>Carbon tetrachloride</td>
<td>$\beta = 0.30^{i,f}$</td>
</tr>
<tr>
<td><img src="image4.png" alt="image" /></td>
<td>Carbon tetrachloride</td>
<td>$\alpha = 0.53^{k,f}$</td>
</tr>
<tr>
<td><img src="image5.png" alt="image" /></td>
<td>Carbon tetrachloride</td>
<td>$\beta = 0.13^{i,f}$</td>
</tr>
<tr>
<td><img src="image6.png" alt="image" /></td>
<td>Carbon tetrachloride</td>
<td>$\beta = 0.20^{m,f}$</td>
</tr>
<tr>
<td><img src="image7.png" alt="image" /></td>
<td>Carbon tetrachloride</td>
<td>$\beta = 0.23^{m,f}$</td>
</tr>
<tr>
<td><img src="image8.png" alt="image" /></td>
<td>Carbon tetrachloride</td>
<td>$\beta = 0.31^{m,f}$</td>
</tr>
<tr>
<td><img src="image9.png" alt="image" /></td>
<td>Benzene</td>
<td>$\alpha = 0.33^{r}$</td>
</tr>
<tr>
<td><img src="image10.png" alt="image" /></td>
<td>Benzene</td>
<td>$\alpha = 0.44^{n}$</td>
</tr>
<tr>
<td><img src="image11.png" alt="image" /></td>
<td>Benzene</td>
<td>$\alpha = 0.69^{r}$</td>
</tr>
</tbody>
</table>
### TABLE I (continued)

<table>
<thead>
<tr>
<th>Hydrogen bond</th>
<th>Solvent</th>
<th>Brønsted slope $(\alpha$ or $\beta)^a$</th>
</tr>
</thead>
</table>
| \[
\begin{array}{ccc}
X & \text{OH} \cdot \text{O} & \text{N} \\
\text{OH} & \text{N} & \text{CH}_3
\end{array}
\] | Benzene | $\alpha = 0.30^a$ |
| \[
\begin{array}{ccc}
X & \text{OH} \cdot \text{N} & \text{H}
\end{array}
\] | Benzene | $\alpha = 0.28^a$ |

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\(a\) $\alpha$ and $\beta$ values are slopes of plots of log $K$ for hydrogen bond formation against the aqueous $pK_a$ values of the hydrogen bond donor or acceptor. In cases with multiple potential hydrogen bond donors and acceptors, complexes are drawn for simplicity of representation, although the functional groups involved in the hydrogen bond have not been determined in general. The number of data points $(n)$ and correlation coefficient $(r)$ for each Brønsted plot are indicated in the parentheses after each reference. The range of $\beta$ values observed in various model studies is not rationalized easily and suggests that we do not yet have a complete account of the factors that affect the energetics of hydrogen bonding. Tables I and II include all the examples of Brønsted slopes for hydrogen bonding of which we are aware. We would appreciate communication of additional examples for inclusion in future compilations.

\(b\) Reference 19 ($n = 8$, $r > 0.99$ for the $\alpha$ value; $n = 11$, $r = 0.77$ for the value of $\beta$).

\(c\) Reference 1 ($n = 11$, $r > 0.99$).

\(d\) Reference 3 ($n = 6$, $r > 0.99$ for the hydrogen bond in phthalate monoanions; $n = 14$, $r > 0.99$ for the hydrogen bond between substituted phenols and 3,4-dinitrophenolates).


\(f\) Individual equilibrium constants for hydrogen bond formation from the cited reference were used to construct Brønsted plots and obtain the $\alpha$ and $\beta$ values listed.

\(g\) G. Albrecht and G. Zundel, *Z. Naturforsch.* 39a, 986 (1984) ($n = 6$, $r = 0.94$).


\(i\) Reference 52 ($n = 7$, $r > 0.99$ for the hydrogen bond with substituted alcohols; $n = 13$, $r = 0.90$ for hydrogen bonding with substituted phenols; $n = 8$, $r = 0.97$ for hydrogen bonding with substituted carboxylic acids).


\(k\) J. Rubin, B. Z. Senkowski, and G. S. Panson, *J. Phys. Chem.* 68, 1601 (1964) ($n = 8$, $r = 0.99$).

\(l\) J. Rubin and G. S. Panson, *J. Phys. Chem.* 69, 3089 (1965) ($n = 8$, $r = 0.99$).


local solvent or environment on the electrostatic interaction between nearby charged or dipolar groups. Thus, unlike the bulk dielectric constant, which represents the ability of a solvent to screen electrostatic interactions between groups distant from one another, the effective dielectric depends on the molecular interactions surrounding the hydrogen bond donors and acceptors. As described in this section, the low effective dielectric of enzymatic interiors may allow active site hydrogen bonds with substrate moieties undergoing charge rearrangement to provide a substantial catalytic contribution.\textsuperscript{54–60} This effect has been demonstrated in a model study in which the dependence of the equilibrium for hydrogen bond formation ($K^{\text{HB}}$) on the $pK_a$ of the hydrogen bonding groups was compared directly in water and in an organic solvent for the same compounds, a series of substituted salicylate monoanions.\textsuperscript{1} The method used in this study to isolate the equilibrium for hydrogen bond formation is first described, as this approach may be applicable for addressing other aspects of hydrogen bonding systems.\textsuperscript{61} Results of the model study, the implications for enzymatic catalysis, and relevant enzymatic results are then described.

\textbf{METHOD FOR LINEAR FREE ENERGY ANALYSIS OF HYDROGEN BOND STRENGTH FOR INTRAMOLECULAR HYDROGEN BONDS.} Equilibria for formation of the hydrogen bond between the adjacent carboxylate and hydroxyl groups in salicylate monoanions (Structure 1) were determined according to Scheme 3. Formation of this hydrogen bond stabilizes the salicylate monoanion relative to the neutral salicylic acid, resulting in a decrease in the observed $pK_a$ ($pK_a^{\text{obsd}}$) of this compound. Although the value of ($pK_a^{\text{obsd}}$) in water is simply determined using a standard pH electrode, determinations of $pK_a$ values in organic solvents are more involved. Fortu-

\textsuperscript{59} The analyses described in this article use model systems to isolate the energetic effects from hydrogen bonds to groups undergoing charge rearrangement. Solvents with low dielectrics and/or low effective dielectrics have been used to model the expected low effective dielectric environment of enzyme active sites, as described in the text. Nevertheless, it should be recognized that the overall energetic interactions with a transition state must be favorable on an enzyme relative to the interactions in aqueous solution in order for the enzyme to provide catalysis. Thus, any potential penalty from imbedding a transition state within a low effective dielectric active site must be overcome by other favorable factors such as additional hydrogen bonds, additional electrostatic interactions, and positioning effects (Refs. 2, 4, 60, and references therein).
nately, indicator scales have been established for $pK_a$ values in dimethyl sulfoxide (DMSO) by Bordwell and co-workers, allowing $pK_a$ values to be obtained spectrophotometrically from the absorbance of proton indicator dyes (Refs. 62–64 and references therein).

Electron-donating and electron-withdrawing substituents on the salicylic acid ($-X$ in Scheme 3) change the charge distribution of the hydrogen bond donor and acceptor. The substituents alter the ability of the hydroxyl and carboxylate groups to donate and accept a hydrogen bond, resulting

\[ K_{\text{HB}}^{\text{obsd}} = K_{\text{obsd}}^{\text{int}} / K_{\text{a}}^{\text{int}} \]

\[ K_{\text{a}}^{\text{int}} \approx K_{\text{a}}^{\text{int}'} \]

**Scheme 3**

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in a change in the equilibrium for hydrogen bonding ($K^{HB}$); this is the effect that we want to follow. However, the substituents also have a second effect on ($pK^\text{obsd}_a$) of the carboxylic acid group, altering its "intrinsic" $pK_a$ ($pK^\text{int}_a$). The intrinsic $pK_a$ is a hypothetical equilibrium that represents the $pK_a$ the carboxylic acid group would have in the absence of the hydrogen-bonding interaction. Thus, the effect on $pK^\text{int}_a$ reflects the "classical" substituent effect and can be approximated by the $pK_a$ of the corresponding compound in which the hydroxyl group is para to the carboxylic acid instead of ortho (Scheme 3B, $pK^\text{int}_a \approx pK^\text{int'}_a$). Ortho and para substituents have similar inductive and resonance effects, but the para positioning prevents intramolecular hydrogen bonding between the $-\text{COO}^-$ and $-\text{OH}$ groups. As depicted in Scheme 3, the difference between the intrinsic and observed $pK_a$ of the $-\text{COOH}$ group provides a measure of the equilibrium for formation of the hydrogen bond$^{1,65}$:

$$K^{HB} = \frac{K^\text{obsd}_a}{K^\text{int}_a} \quad \text{and} \quad \log K^{HB} = pK^\text{int}_a - pK^\text{obsd}_a$$

**Results and Implications.** Measuring the equilibrium constant for hydrogen bond formation for salicylate monoanions with a series of substituents in water and in DMSO allowed the determination of linear-free energy relationships for hydrogen bonding in these two different environments (Fig. 3). The dependence of the hydrogen-bonding equilibrium on $\Delta pK_a$, the difference between the $pK_a$ values for the hydrogen bond donor ($-\text{OH}$) and acceptor ($-\text{COO}^-$), is steeper in DMSO (Fig. 3, circles) than

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$^{65}$ It should be noted that this method does not provide a perfect measure of these hydrogen-bonding equilibria. This is both because of the difficulties in isolating the "absolute" or "intrinsic" energy for hydrogen bonds, as discussed in the text, and because the following simplifying assumptions were made in this analysis: (1) Ortho- and para-OH groups have similar "intrinsic" effects on deprotonation of the $-\text{COOH}$ group; (2) the steric effect on the $-\text{COOH}$ $pK_a$ from the ortho-OH group is minimal; and (3) the inductive effects from the ortho-OH group and added substituents ($-X$ in Scheme 3) are independent and therefore additive. These assumptions could lead to small deviations in the estimated "intrinsic" $pK_a$ values of the hypothetical nonhydrogen-bonded species (Scheme 3A, bottom species) and thus small effects on the $K^{HB}$ values obtained. Nevertheless, control experiments suggest that these assumptions hold to a first approximation (Refs. 1 and 2 and references therein). More importantly, the analysis was performed for a structurally homologous series of compounds so that deviations caused by these simplifications are expected to largely cancel, allowing the change in the energetics of these hydrogen bonds to be determined reliably. Thus, these approximations are not expected to affect the conclusions from this study.
in water (Fig. 3, squares), with Brønsted slopes of $\beta = 0.73$ and 0.05, respectively.\textsuperscript{66}

The steeper Brønsted slope in DMSO than in water suggests that decreasing the effective dielectric of the media can provide a greater strengthening of hydrogen bonds that accompanies an increase in the charge density on the donor/acceptor groups. Although we know of no other direct comparison between hydrogen bonds in aqueous and nonaqueous environments, steep Brønsted slopes for both intramolecular and intermolecular hydrogen bonds are often observed in nonaqueous solvents and in the gas phase (Tables I and II), suggesting that the observation described earlier is general.

Enzymes may provide an active site environment with a lower effective dielectric than water because protein interiors contain many nonpolar side chains that cannot stabilize isolated charges effectively and because the charged and polar groups that are present are typically involved in networks of interactions that limit their ability to rearrange and stabilize isolated charges (see Refs. 1, 54–58, and references therein for more detailed discussions). Analogous to the enzymatic active site, DMSO molecules are not effective at stabilizing negative charges: their methyl groups prevent close

\textsuperscript{66} In Fig. 3, a common $pK_a$ scale in water is used to allow direct comparison of the magnitude of changes in the equilibrium for hydrogen bond formation in DMSO and in water for the same series of compounds. Using a common $\Delta pK_a$ scale allows simple representation of the transition state and ground state in terms of $\Delta pK_a$ values that remain constant, regardless of solvent. This is illustrated in the following example. Consider two compounds in Fig. 3 with $\Delta pK_a$ values of 0 and 5 in water, respectively. This five-unit difference in the aqueous $\Delta pK_a$ value of the two compounds ($\Delta \Delta pK_a$) is analogous to a change in the $pK_a$ value of a substrate group in the course of a reaction. Using the Brønsted slopes of $\beta_{\text{DMSO}} = 0.73$ and $\beta_{\text{water}} = 0.05$ in Fig. 3 that are based on the aqueous $pK_a$ scale, the difference in the strength of the two hydrogen bonds in DMSO and in water can be simply compared as $\Delta \Delta \log K^{\text{HB}} = \Delta \Delta pK_a \times (\beta_{\text{DMSO}} - \beta_{\text{water}}) = 5 \times (0.73 - 0.05) = 3.4$. However, because the $pK_a$ scale in DMSO is 2.4-fold larger than that in water, the Brønsted slope for the hydrogen bond based on such a $pK_a$ scale would be smaller, with $\beta_{\text{DMSO}} = 0.30$; for the same reason, the change in the $\Delta pK_a$ value would be larger, with $\Delta \Delta pK'_a = 12$ for the same two compounds. The difference in the strength of the two hydrogen bonds in DMSO and water is then compared as $\Delta \Delta \log K^{\text{HB}} = \Delta \Delta pK'_a \times \beta_{\text{DMSO}} - \Delta \Delta pK_a \times \beta_{\text{water}} = 12 \times 0.30 - 5 \times 0.05 = 3.4$. The same result is obtained using both scales, as it should, but the Brønsted slopes obtained from a common $pK_a$ scale in water allows the change in hydrogen bond strength for the two compounds to be compared directly in different solvents, without the necessity to separately correct for the change in the $pK_a$ scale in going from water to DMSO. It should also be noted that the slope of 0.73 in DMSO does not represent the apparent degree of proton transfer in the hydrogen bond, as described earlier for the $\beta$ values in water. Such interpretations require the use of the $pK_a$ scale for the solvent in which the hydrogen-bonding equilibrium is being determined and have sometimes been made incorrectly in the literature.
Fig. 3. A greater strengthening of the hydrogen bond accompanying changes in the charge density of donor/acceptor groups in DMSO than water. The equilibrium for formation of the hydrogen bond ($K_{f}^{\text{HB}}$) in a series of substituted salicylate monoanions is plotted against $\Delta pK_a$, the difference in the $pK_a$ value of the hydrogen bond donor and acceptor. To allow direct comparison of the magnitude of changes in $K_{f}^{\text{HB}}$ accompanying changes in the charge distribution of the donor/acceptor groups, a common $pK_a$ scale in water was used for the hydrogen bond in both media. Adapted from S. Shan and D. Herschlag, *Proc. Natl. Acad. Sci. U.S.A* 93, 14474 (1996), with permission.

approach of the partially positively charged sulfur to anions and their large size limits rearrangement of the molecules in the solvation shell. DMSO may therefore be a reasonable, albeit crude, mimic of the active site environment for interactions of negatively charged species with hydrogen bond donors (see also Ref. 1 and references therein). Data from model studies and the apparent low effective dielectric of enzymatic active sites suggest that hydrogen bonds can be strengthened in the transition state to a greater
TABLE II
PARTIAL LIST OF OBSERVED BRØNSTED SLOPES FOR HYDROGEN BONDING IN THE GAS PHASE

<table>
<thead>
<tr>
<th>Hydrogen bond</th>
<th>Brønsted slope (α or β) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X-OH^*F^-$ (X=H, R)</td>
<td>$\alpha = 0.51^{b}$</td>
</tr>
<tr>
<td>$HOH^*O-X$ (X=H, R)</td>
<td>$\beta = 0.28^{d}$</td>
</tr>
<tr>
<td>$X-OH^*OCC-R$ (X=H, R)</td>
<td>$\alpha = 0.34^{d}$</td>
</tr>
<tr>
<td>$X-OH^*Cl^-$ (X=H, R)</td>
<td>$\alpha = 0.17^{e,f}$</td>
</tr>
<tr>
<td>$X-R_2NH^*OH_2$</td>
<td>$\alpha = 0.26^{e,f}$</td>
</tr>
<tr>
<td>$X-R_2NH^*NH_3^+$</td>
<td>$\alpha = 0.30^{e,f}$</td>
</tr>
<tr>
<td>$CH_3NH_3^*O-X$</td>
<td>$\beta = 0.17^{e,f}$</td>
</tr>
<tr>
<td>$CH_3NH_3^*O=\overset{R_1}{\makebox[0pt][r]{R_2}}$</td>
<td>$\beta = 0.51^{e,f}$</td>
</tr>
<tr>
<td>$X-\overset{\text{NH}^*}{\text{H}_2O}$</td>
<td>$\alpha = 0.15^{e,f}$</td>
</tr>
<tr>
<td>$X-OH_2^*OH_2$</td>
<td>$\alpha = 0.34^{e,f}$</td>
</tr>
<tr>
<td>$R_1\overset{\text{OH}^*}{\text{OH}_2}$</td>
<td>$\alpha = 0.31^{e,f}$</td>
</tr>
</tbody>
</table>

(continued)

extent within the context of the enzymatic active site than in aqueous solution.$^{59}$

This mechanism may provide substantial rate enhancements relative to solution reactions. The potential catalytic effect from the interaction of His-95 with the substrate carbonyl oxygen in the TIM reaction is outlined in Fig. 4, and a crude estimate for the magnitude of this effect is obtained as follows.$^{67}$ As the reaction proceeds from the ground state to the transition state, the $pK_a$ of the carbonyl oxygen increases by $\sim$10 units.$^{68}$ If the Brønsted slopes of 0.7 and 0.05 observed for DMSO and water in model

---

$^{67}$ The stronger hydrogen bond donating ability of Lys-12$^+$ and the lower effective dielectric of the enzyme active site could both contribute to a steeper Brønsted slope for the $C=O \cdot \text{Lys-12}^+$ hydrogen bond. For discussion of the catalytic contribution of hydrogen bonds arising from the lower effective dielectric of the enzyme active site, the $C=O \cdot \text{His}^{89}$ hydrogen bond is chosen instead because the neutral histidine and water have more similar $pK_a$ values ($\sim$14 and 16, respectively).

### TABLE II (continued)

\(^{a}\) \(\alpha\) and \(\beta\) values are slopes of plots of the enthalpy for hydrogen bond formation (\(\Delta H_{\text{HB}}^\text{f}\)) against the enthalpy for gas phase deprotonation (\(\Delta H_{\text{HB}}^\text{d}\)) of the hydrogen bond donor (for \(\alpha\) values) or acceptor (for \(\beta\) values). The number of data points (\(n\)) and correlation coefficient (\(r\)) for each Brønsted plot are indicated in the parentheses after each reference. Significant deviations from a linear relationship are observed in some correlation of \(\Delta H_{\text{HB}}^\text{f}\) vs \(\Delta H_{\text{HB}}^\text{d}\). A cutoff of \(r \geq 0.90\) was used in selecting data sets. In addition, a slope of \(>1\) was also observed for one set of data in reference \(g\) (not shown). These deviations suggest that factors in addition to simple electrostatics affect the energetics of complex formation in these data sets.

\(^{b}\) Reference 12 (\(n = 12, r > 0.99\)).

\(^{c}\) J. E. Mihalick, G. G. Gatev, and J. Brauman, J. Am. Chem. Soc. 118, 12424 (1996) (\(n = 13, r > 0.99\)).

\(^{d}\) M. Mautner and L. W. Sieck, J. Am. Chem. Soc. 108, 7525 (1986) (\(n = 4, r > 0.99\) for the HOH \(\cdot \) \(\cdot \) \(\cdot \) X hydrogen bond; \(n = 7, r = 0.99\) for the X \(- \) OH \(\cdot \) \(\cdot \) OOC \(- \) R hydrogen bond).

\(^{e}\) R. Yamdagni and P. Kebarle, J. Am. Chem. Soc. 93, 7139 (1971) (\(n = 4, r = 0.96\)).

\(^{f}\) Individual enthalpies for hydrogen bond formation from the cited reference were used to construct Brønsted plots and to obtain the \(\alpha\) and \(\beta\) values listed.

\(^{g}\) M. Meotner, J. Am. Chem. Soc. 106, 1257 (1984) (\(n = 9, r = 0.99\) for the X \(- \) R\(_2\)NH\(^+\) \(\cdot \) OH\(_2\) hydrogen bond; \(n = 5, r = 0.98\) for the X \(- \) R\(_2\)NH\(^+\) \(\cdot \) NH\(_3\) hydrogen bond; \(n = 7, r = 0.89\) for the hydrogen bond of methylamine with substituted alcohols; \(n = 4, r = 0.96\) for the hydrogen bond of methylamine with ketones; \(n = 8, r = 0.93\) for the hydrogen bond of substituted pyridines with water; \(n = 3, r = 0.99\) for the hydrogen bond of substituted hydronium ions with water; \(n = 5, r = 0.96\) for the hydrogen bond of substituted oxonium ion with water).

Studies were to hold for the enzymatic and solution hydrogen bonds, respectively (Fig. 4, \(\beta_{\text{soln}}\) and \(\beta_{\text{E}}\) in the top and bottom reactions, respectively), this interaction would provide a rate enhancement of \(\sim 10^6\)-fold \([\text{rate enhancement} = 10^{\Delta \beta \log K_a, (\beta_{\text{soln}} \times \beta_{\text{E}}) = 10^{\Delta \log 0.7 \times 0.05} \approx 10^5}; \Delta \Delta G^\dagger = \Delta \Delta G^\text{E} - \Delta \Delta G^\text{soln} \approx 9 \text{kcal/mol (Fig. 4)}\]). This estimate certainly oversimplifies features of the active site and ignores the potential change in \(\beta\) value in going from the formally neutral carbonyl oxygen in the ground state to the partially charged enolate-like oxygen in the transition state. Also, DMSO is an imperfect mimic of the active site environment, as noted earlier. Nevertheless, the analysis suggests that the steeper Brønsted slopes in environments of low effective dielectric and the large change in the charge...
Fig. 4. Potential catalytic contribution from a greater strengthening of hydrogen bonds accompanying charge redistribution in a low dielectric enzyme active site than in aqueous solutions depicted for the example of the TIM reaction. Reproduced from S. Shan and D. Herschlag, *Proc. Natl. Acad. Sci. U.S.A* 93, 14474 (1996), with permission.

Distribution on substrate groups in the course of a reaction allow a substantial amount of catalysis to be obtained from hydrogen-bonding interactions.\(^\text{59}\)

**Do Enzymes Exhibit the Large Increase in Hydrogen Bond Strength upon Charge Rearrangement That Is Suggested from Results in Model Systems?** An enzymatic study that addresses this question used a series of unnatural amino acids, fluoro-substituted tyrosines, to...
obtain a Brønsted $\beta$ value for a Tyr·Glu$^-$ hydrogen bond in staphylococcal nuclease.\textsuperscript{69} The effect of the fluoro-substituted tyrosines (Fn-Tyr) on the stability of the folded enzyme was determined. Analysis of the results in a manner analogous to that outlined in Scheme 3, Fig. 4, and the previous discussion suggests that the $\beta$ value is 0.35–0.75 larger for the Fn-Tyr$^{\text{OH}}$. Glu$^-$ hydrogen bond on the enzyme than the Fn-Tyr$^{\text{OH}}$·OH$_2$ hydrogen bond in solution,\textsuperscript{1} consistent with the expectation from the model studies. An analysis of the binding of a series of phenolate ions to the 3-oxo-$\Delta^5$-steroid isomerase active site suggested a smaller increase in the $\beta$ value, of $\sim$0.1–0.2, for the hydrogen bond(s) at the active site relative to solution.\textsuperscript{70} Clearly, more quantitative studies will be required in order to establish the magnitude and range of perturbation of $\beta$ values on proteins. Advances in the ability to incorporate unnatural amino acids into proteins should allow more studies of this type to be carried out in the near future and to be correlated with structural effects.\textsuperscript{71–73}

Rate Enhancements from Multiple Hydrogen Bonds at Sites of Charge Rearrangement. The catalytic contribution of a single hydrogen bond with a substrate group undergoing charge rearrangement in the course of a reaction was discussed in the previous section. However, enzymatic active sites have many groups that interact with substrates, and typically there are multiple interactions with the groups that undergo charge rearrangement. The two hydrogen bonds from His-95 and Lys-12 to the substrate carbonyl oxygen in the active site of TIM provide one example\textsuperscript{17} and the two hydrogen bonds in the oxyanion hole of serine proteases provide another example (Ref. 74 and references therein; for some additional examples, see Refs. 75–80 and references therein). Hence the question arises: What are the energetic consequences of multiple hydrogen bonds? A model study\textsuperscript{2} is described that suggests that the energetic effects of multiple hydrogen bonds can be large and additive for donor and acceptor groups that are prepositioned with respect to each other, as occurs in an enzymatic active site.

The monoanion of 2,6-dihydroxybenzoic acid (II), in which the carboxylate group can be hydrogen bonded to both of the ortho-hydroxyl groups, was used to mimic multiple hydrogen-bonding interactions at an enzymatic active site. The combined energetic effect of the two hydrogen bonds in DMSO was isolated by the same approach outlined in Scheme 3 for the hydrogen bond in salicylate monoanions. After accounting for inductive effects, a stabilization from the two hydrogen bonds (K_HB, Scheme 3) of 10¹⁰-fold or ΔΔG_HB = 14.4 kcal/mol in DMSO was obtained. This is close to the value of 15.8 kcal/mol expected for the additive effect of two \(-\text{OH} \cdot \text{COO}^-\) hydrogen bonds. The value of 15.8 kcal/mol was obtained from the estimated stabilization from a single hydrogen bond in salicylate monoanion (I) of 7.9 kcal/mol.

The near additivity of the energetics of hydrogen bonds in II suggests that there is no substantial charge redistribution at the donor and acceptor groups upon formation of one hydrogen bond. An additive effect would not be expected for hydrogen bonds that are predominantly covalent in nature, as extensive electronic rearrangement in a group upon forming a covalent bond would be expected to weaken the ability of this group to form a second hydrogen bond. Thus, the nearly additive hydrogen bond energies in the 2,6-dihydroxybenzoate monoanion (II), combined with the comparisons presented in the previous section, suggest that large energetic contributions can be obtained from hydrogen bonds that are largely electrostatic in nature in nonaqueous environments and, presumably, in enzyme active sites (see also Refs. 1–3 and references therein for detailed discussions).

\[\begin{align*}
\text{I} & \quad \text{O} \cdot \text{O} \cdot \text{O} \\
\text{II} & \quad \text{O} \cdot \text{O} \cdot \text{O} \\
\text{III} & \quad \text{O} \cdot \text{O} \cdot \text{O}
\end{align*}\]

Can Geometrical Changes in Going from the Ground State to the Transition State Lead to More Favorable Transition-State Hydrogen Bonding? Geometrical effects on the energetics of hydrogen bonds can be demonstrated by comparing the energetics of hydrogen bonds in salicylate (I) vs phthalate (III) monoanions. Despite the greater acidity of the carboxylic acid group in III relative to the hydroxyl group in I and the expectation of a stronger hydrogen bond from the carboxylic acid, the hydrogen bonding
in phthalate monoanion (III) is 2.7 kcal/mol less favorable than that in salicylate monoanion (I). This suggests that the geometry of the carboxylic acid and carboxylate groups in III is suboptimal for hydrogen bonding.1,3

Analogously, geometrical constraints within an $E \cdot S$ complex might be used to weaken hydrogen-bonding interactions in the ground state; geometrical changes in the course of the reaction might allow the hydrogen bond to be strengthened in the transition state, thereby providing a rate enhancement. For example, it has been suggested that the $C=O$ bond is too short for optimal hydrogen bonding in the oxyanion hole of serine proteases, so that optimal hydrogen bond distances are only achieved when the $C=O$ bond is lengthened in the transition state.4,81–84

Catalysis via geometrical destabilization of hydrogen bonds in the ground state is conceptually distinct from catalysis via strengthening of hydrogen bonds accompanying charge rearrangement in the course of a reaction. In practice, however, these two mechanisms are difficult to dissect because geometrical changes typically occur in concert with charge rearrangement. In addition, even though geometrical effects are observed in model compounds, the geometrical constraints imposed by the noncovalent interactions of an enzyme are presumably less severe than those imposed by covalent interactions in model compounds. The extent to which enzymes can "recognize" the geometrical changes of the substrate and thereby preferentially stabilize the transition state remains to be determined.

Interconnections between Binding Interactions and the Differential Strengthening of Hydrogen Bonds

The previous sections focused on hydrogen bonds with substrate moieties undergoing charge rearrangement in the course of a reaction and suggested that the increased strengthening of hydrogen bonds in the enzymatic active site relative to aqueous solution may provide substantial rate enhancements. However, two features of the enzymatic active site are required to achieve catalysis via this mechanism. First, the enzymatic and substrate hydrogen bond donors and acceptors must be aligned with respect to one another. Second, the enzymatic active site must provide a low effective dielectric environment for these hydrogen bonds.

Fixation of active site groups with respect to each other and with respect to bound substrate can also lower the entropic barrier in going from the ground state to the transition state,\textsuperscript{4,18} a mechanism that was not discussed earlier. For example, in the active site of TIM, hydrogen bond donors and acceptors and the general acid and base are positioned in the active site with respect to the bound substrate.\textsuperscript{17} This minimizes the reorganization of solvent that confers a substantial entropic barrier in nonenzymatic reactions.\textsuperscript{85}

Catalysis via lowering the entropic barrier of a reaction is conceptually distinct from catalysis via the greater strengthening of hydrogen bonds accompanying charge rearrangement in the enzymatic active site relative to aqueous solution. However, these mechanisms are inextricably linked in practice because both are effected through the rigidity and precise alignment of the active site groups with respect to each other and with respect to the substrate groups. Positioning of active site residues within the folded enzyme both aligns functional groups for interactions with substrates and lowers the local dielectric.\textsuperscript{1,4,18,54–58} Conversely, the “catalytic” hydrogen bonds to groups undergoing charge rearrangement in the course of reaction also contribute to rigidifying the active site and positioning the substrate with respect to other catalytic groups.

This interconnection between binding interactions and interactions at positions involved in chemical transformation represents a fundamental property of enzymatic catalysis (Refs. 4 and 86 and references therein). However, this interconnection also limits our ability to experimentally dissect the catalytic contributions from each mechanism. The following example illustrates these interconnections.

In the active site of TIM, Tyr-208 is hydrogen bonded to a residue in the flexible loop of TIM. Although Tyr-208 is not in direct contact with the substrate, mutation of this residue to phenylalanine results in >2000-fold reduction in $k_{cat}/K_m$.\textsuperscript{87,88} This mutation presumably disrupts the network of interactions within the active site, thereby impairing the interaction of catalytic residues with the substrate (Fig. 5). For example, the active site general base, Glu-65, may be misaligned with respect to the substrate reaction center. This mutation may also impair the interaction of the substrate phosphate group with a number of residues in the flexible loop that are used to position the substrate. It is also possible that the active site becomes more solvated when the closure of the loop is impaired, resulting

\begin{itemize}
  \item \textsuperscript{87} N. S. Sampson and J. R. Knowles, \textit{Biochemistry} \textbf{31}, 8482 (1992).
  \item \textsuperscript{88} N. S. Sampson and J. R. Knowles, \textit{Biochemistry} \textbf{31}, 8488 (1992).
\end{itemize}
Fig. 5. Schematic representation of active site interactions of TIM complexed with a transition-state analog, phosphoglycolohydroxamate (PGH). The active site base, Glu-165, interacts with N1 of PGH, which represents the site of proton abstraction in the substrate. A loop consisting of residues 168–178 (dark ribbon) folds down over the active site on substrate binding and interacts with the phosphate of PGH. To show the connection between this loop and Glu-165, the backbone of the loop is extended to residue 165 (lighter ribbon). Tyr-208 is important for closure of this loop via hydrogen bonding with the backbone of Ala-176 (see text). Hydrogen bonds from His-95 and Lys-12 to the carboxyl oxygen of PGH discussed in the text are also included. Oxygen atoms are shown in black, nitrogen atoms in light gray, carbon atoms in dark gray, and the phosphorus atom of PGH is shown in white.

in increased effective dielectric of the active site. This could lessen the catalytic contribution of hydrogen bonds with substrate moieties undergoing charge relocalization, such as those from His-95 and Lys-12.

The comparative approach described in the previous sections allows probing of the potential energetic contribution of hydrogen bonds that stems from electrostatic properties. These effects are probed more readily in model systems than on enzymes because of the limitations described earlier. Nevertheless, the application of linear free energy relationship to proteins using unnatural amino acids may minimize structural perturbations, thereby providing an improved, although still imperfect, means to
probe aspects of the catalytic contribution of hydrogen bonds. The ability to introduce precise and conservative modifications is crucial for preserving the role of these hydrogen bonds in positioning while systematically varying the electrostatic properties of the hydrogen bond. It will also be important to obtain structural and spectroscopic information on these modified enzymes to complement and expand on the results from energetic and mechanistic analyses and to better understand the physical nature of hydrogen bonds.\(^{21}\)

Summary

Enzymes can provide catalysis by increasing the strengthening of hydrogen bonds to groups undergoing charge rearrangement in the course of reaction relative to the strengthening of the hydrogen bonds in the corresponding solution reactions. This can be accomplished by using hydrogen bond donors and acceptors that are stronger than water and by lowering the effective dielectric relative to that in aqueous solution. We suggest that these electrostatic effects are of general significance in enzymatic catalysis.

The effective dielectric is lowered by the overall "rigidity" of the folded enzyme, which facilitates the formation of active site interactions, and by the fixation of active site functional groups within the enzyme-substrate complex. This underscores the fundamental interconnection of catalytic mechanisms in enzymatic catalysis.

Appendix

The Hine equation [Eq. (8)] describes quantitatively hydrogen bonding between solutes in water as a competition between solute·solute interactions and solute·solvent interactions [Eq. (9)], based on an electrostatic model for hydrogen bonding.\(^{19,45,52,53}\) The Brønsted slopes, \(\alpha\) and \(\beta\), are obtained from the Hine equation by taking the derivative of \(\log K^{\text{HB}}\) with respect to the \(pK_a\) of the hydrogen bond donor or acceptor, respectively, as shown in Eqs. (10a) and (10b). As noted in the text, the \(\alpha\) and \(\beta\) values are slopes that describe the observed dependence of \(\log K^{\text{HB}}\) on the \(pK_a\) of the hydrogen bond donor and acceptor. As shown in Eq. (11), the change in these slopes (i.e., the change in the dependence of \(\log K^{\text{HB}}\) on the acceptor with different hydrogen bond donors and the converse) is described in the Hine model by the interaction coefficient, \(\tau\). The term "\(\log (2 \times 55)\)" in the Hine equation [Eq. (8)] is a statistical correction to account for the competition for hydrogen bonds from 55 M water relative to a 1 M standard state for the solute molecules.
This description can be related to the Coulombic model of Eqs. (4) and (5) because the pKa values in Eq. (8) are analogous to the partial charges on donors and acceptors in Eq. (5). The interaction coefficient \( \tau \) is analogous to the constant in the Coulombic equation [cf. "C" in Eq. (5) and \( \tau \) in Eq. (8)] and is dependent on the distance between the groups and the dielectric of the environment surrounding the hydrogen bonding groups:

\[
\log K_{\text{HB}} = \tau(pK_a^{\text{HOH}} - pK_a^{\text{HA}})(pK_a^{\text{B}} - pK_a^{\text{H}_2\text{O}}) - \log(2 \times 55) \tag{8}
\]

\[
\begin{align*}
H_2O \cdot \text{HA} + B \cdot \text{HOH} &\rightarrow K_{\text{HB}} B \cdot \text{HA} + H_2O \cdot \text{HOH} \tag{9}
\end{align*}
\]

\[
\alpha = \frac{\partial \log K_{\text{HB}}}{\partial pK_a^{\text{HA}}} = \tau(pK_a^{\text{B}} - pK_a^{\text{H}_2\text{O}}) \tag{10a}
\]

\[
\beta = \frac{\partial \log K_{\text{HB}}}{\partial pK_a^{\text{B}}} = \tau(pK_a^{\text{HOH}} - pK_a^{\text{HA}}) \tag{10b}
\]

\[
\tau = \frac{\partial \alpha}{\partial pK_a^{\text{B}}} = \frac{\partial \beta}{\partial pK_a^{\text{HA}}} \tag{11}
\]

For the reaction catalyzed by TIM (Fig. 1), the increase in the Brønsted slope for the hydrogen bond with the active site Lys-12 relative to the hydrogen bond with water (\( \Delta \beta \)) can be calculated as

\[
\Delta \beta = \beta^{\text{Lys}} - \beta^{\text{HOH}} = \tau \times (pK_a^{\text{HOH}} - pK_a^{\text{Lys}}) = 0.013 \times (16 - 9) \approx 0.1.
\]

This calculation uses the value of \( \tau = 0.013 \) observed in aqueous solution\(^{19,50,51} \) and assumes that the relative strength of the hydrogen bonds from Lys\(^+ \) and water (HOH) can be estimated from the difference in their proton affinities (i.e., pK\(_a\) values). This value of \( \Delta \beta = \beta^{\text{Lys}} - \beta^{\text{HOH}} \approx 0.1 \) and the change of \( \sim 10 \) units in the pK\(_a\) of the carbonyl oxygen in going from the ground state to the transition state\(^{68} \) allows estimation of the difference in the magnitude of the increase in the hydrogen-bonding equilibrium in the course of the enzymatic reaction relative to that in solution as

\[
\Delta \Delta \log K_{\text{HB}} = \Delta \log K_{\text{HB}}^{\text{Lys}} - \Delta \log K_{\text{soln}}^{\text{HB}} = \beta^{\text{Lys}} \times \Delta pK_{a}^{\text{GS} \rightarrow \text{TS}} - \beta^{\text{HOH}} \times \Delta pK_{a}^{\text{GS} \rightarrow \text{TS}} = \Delta \beta \times \Delta pK_{a}^{\text{GS} \rightarrow \text{TS}} = 0.1 \times 10 = 1
\]

This gives an estimated rate enhancement of \( \sim 10 \) fold [rate enhancement = \( 10^{\Delta \Delta \log K_{\text{HB}}} = 10 \); \( \Delta \Delta G^+ = \Delta \Delta G^E - \Delta \Delta G^{\text{soln}} = -2.303 RT \times \Delta \Delta \log K_{\text{HB}} = 275 \) \].
LOW-BARRIER HYDROGEN BONDS

−1.4 kcal/mol (Fig. 1). As noted in the text, this effect may be accentuated by the low effective dielectric of the enzymatic active site.

Acknowledgments

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[12] Application of Marcus Rate Theory to Proton Transfer in Enzyme-Catalyzed Reactions

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Introduction

Although the great variety of chemical substances and the wide diversity of their reactions preclude a generally valid relationship between rate and equilibrium constants, it would seem not unreasonable to expect such a correlation to exist for the same reaction of a sufficiently homogeneous family of substrates. Marcus rate theory fulfills this expectation in a particularly simple and intuitively satisfying way by relating the free energy of activation of a chemical reaction, \( \Delta G^\ddagger \), to its overall standard free energy change, \( \Delta G^0 \), using only one other parameter, \( \Delta G^0_\ddagger \), commonly called the intrinsic barrier. This basic relationship of Marcus theory is shown in Eq. (1):

\[
\Delta G^\ddagger = (1 + \Delta G^0/4\Delta G^0_\ddagger)^2 \Delta G^0_\ddagger
\]

(1)

Marcus first developed his theory for electron transfer reactions,\(^1\) but it has been applied widely to proton transfer as well.\(^2\)\textsuperscript{−18} It can be derived

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