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Energetic Effects of Multiple Hydrogen Bonds. Implications for Enzymatic Catalysis

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Abstract: The rate enhancements obtained by enzymes often require greater than 20 kcal/mol of stabilization that is specific to the transition state. Inspection of active sites suggests that enzymes often use multiple interactions with substrate groups undergoing charge rearrangement to help provide this preferential stabilization. We have analyzed the deprotonation, in DMSO, of benzoic acids with one or two hydrogen bond donating substituents *ortho* to the carboxylic acid. This provides a model for the ability of multiple hydrogen bonds to facilitate reactions at enzymatic active sites. The simplicity of this model has allowed the energetic contributions of the hydrogen bonds to be isolated and assessed quantitatively. It was found that one and two hydrogen bonds from the hydroxyl group(s) *ortho* to the carboxyl group provide specific stabilization of the carboxylate relative to the carboxylic acid by 7.9 and 14.4 kcal/mol, respectively. The energetic contribution of the two hydrogen bonds is nearly additive. This provides a quantitative demonstration of the basic principle that the energy of multiple interactions, each of moderate strength, can be combined to make a significant contribution to enzymatic catalysis.

Introduction

It has recently been proposed that low-barrier or short, strong hydrogen bonds (LBHB's) supply a large fraction of the energy required for transition state stabilization by several enzymes.[1] Recent model studies and theoretical calculations suggest that strengthening of a hydrogen bond upon charge rearrangement in the transition state can contribute significantly to enzymatic catalysis. However, there is no evidence for a special energetic contribution from formation of a partially covalent LBHB, and the hydrogen bond energies appear to be less than originally suggested in the LBHB proposals.[2−5]

Instead of relying on a single interaction to provide the bulk of the catalytic power, enzymes can use multiple interactions, with each providing a modest contribution.[6] This widely-recognized principle has been demonstrated in various enzymatic reactions[7] and employed in the design of enzyme inhibitors, synthetic receptors, and catalysts.[8] We describe herein the deprotonation, in DMSO, of benzoic acids with one or two hydrogen bond donating substituents *ortho* to the carboxylic acid. This provides a simple model of the charge rearrangement that occurs during a reaction, allowing a quantitative assessment

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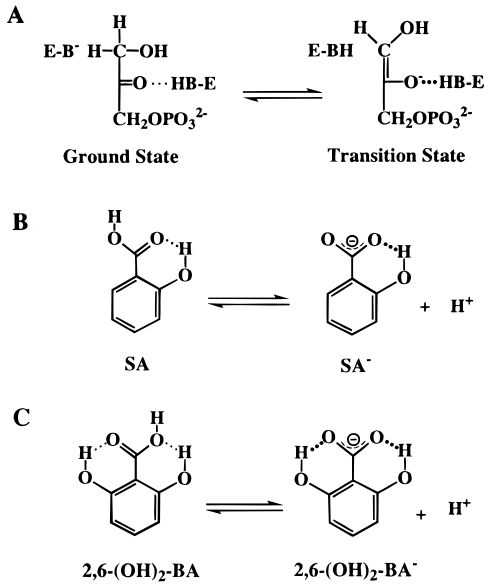
Scheme 1



of the energetic contribution from multiple hydrogen bonds in a nonaqueous environment.

Experimental Design

Typically, charge relocalizes as a reaction proceeds from the ground state to the transition state**.** This is shown for the example of the triose phosphate isomerase reaction in Scheme 1A. The deprotonation of a carboxylic acid can serve as a simple model for a charge rearrangement, in that there is an accumulation of charge on the carboxyl oxygens in the anionic form. The deprotonation of salicylic acid (SA) and related compounds (Scheme 1, B and C) was used herein to mimic charge relocalizations that occur at enzymatic active sites, as these compounds have hydrogen bond donor(s) positioned adjacent to the carboxyl oxygen(s), analogous to active site residues that are positioned to interact with substrate functional groups.

Hydrogen bonding and other electrostatic interactions between enzymatic catalytic groups and the atoms undergoing charge relocalization can be strengthened in the transition state, thereby specifically stabilizing the transition state relative to the ground state and providing a rate enhancement (Scheme 1A, C−O$^-$···HB−E). Similarly, the intramolecular hydrogen bond between the adjacent hydroxyl proton and carboxyl oxygen in SA can be strengthened upon deprotonation of the carboxylic acid (Scheme 1B, COO$^-$···HO).[9] This provides specific stabilization of the monoanion relative to the acid form of the compound (SA$^-$ vs SA), thereby favoring deprotonation, as was previously demonstrated.[11] Here the effect of multiple hydrogen bond donors on this deprotonation was analyzed, using the intramolecular hydrogen bonds between the carboxyl oxygens and the two *o*-hydroxyl groups in 2,6-dihydroxybenzoic acid [2,6-$(OH)_2$-BA, **3**; Scheme 1C]. The energetics of these hydrogen bonds were evaluated in DMSO to crudely mimic hydrogen bonding in a nonaqueous enzymatic active site.[12]

(9) The hydrogen bonds in the acid species are expected to be much weaker than those in the monoanions. Hydrogen bonds involving charged species are typically considerably stronger than those involving neutral species, and there is a strong dependence of hydrogen bond strength on the pK_a's of the hydrogen bond donors and acceptors in organic solvents (refs 3 and 10 and references therein).

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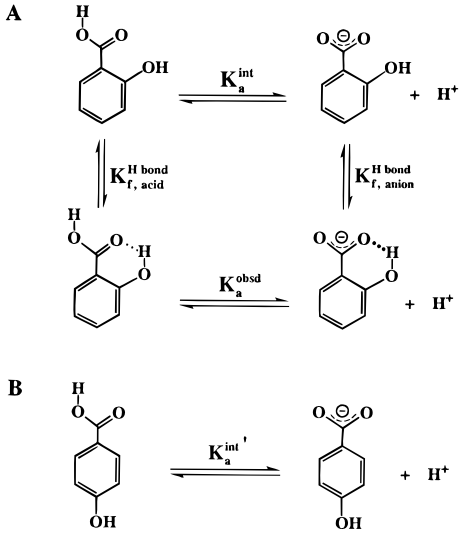
(12) Though DMSO can solvate positively charged species and hydrogen bond donors, it is not effective at stabilizing negative charges. This is because it contains no hydrogen bond donors and its methyl groups limit close approach between its partially positively charged sulfur atom and negatively charged solutes. Further, its large size limits the number of solvent molecules that can be in direct contact with the solute. Since local solvation is limited, DMSO may provide a crude model for aspects of a desolvated active site when considering stabilization of anionic species as in this work, despite its relatively high bulk dielectric constant. In addition, the ability of DMSO to effectively solvate cations [e.g., the free energy of transfer of potassuim ion from water to DMSO is favorable (−3 kcal/mol)[13]] minimizes complications from ion-pairing effects (see Experimental Section).

Table 1. pK_a Determinations in DMSO and the Energetics of Hydrogen Bond(s) Derived from the pK_a Data

compd	pK_a^{obsd} [a]	pK_a^{int} [a]	$-\Delta\Delta G^{H\ bond}$ [c] (kcal/mol)
1	11.08 ± 0.06 (11.1)		
2	6.60 ± 0.05 (6.8)	12.4	7.9
3	3.08 ± 0.10 (3.1)	13.7	14.4 (15.8)[d]
4	12.38 ± 0.06 (12.8)		
5	6.04 ± 0.13[b]	9.80	5.2
6	4.83 ± 0.10	11.1	8.5 (13.1)[d]
7	9.80 ± 0.06[b]		

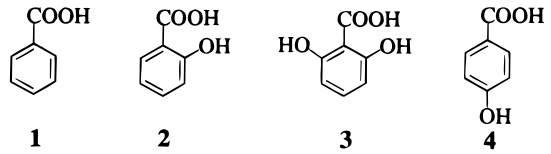
[a] Determined as described in the Experimental Section. Values in parentheses were determined previously. The value for **1** is from refs 13 and 14 and those for **2**, **3**, and **4** are from ref 11. [b] From ref 2. [c] Derived from Scheme 2: $\Delta\Delta G^{H\ bond} = -RT\ \ln(K_{f,anion}^{H\ bond}/K_{f,acid}^{H\ bond}) = -RT\ \ln(K_a^{obsd}/K_a^{int})$, where R is the gas constant (1.987 cal mol^{-1} K^{-1}) and T is temperature in Kelvin (298 K).[15] [d] The value expected if the *ortho* effects are additive (i.e., 15.8 = 7.9 + 7.9 and 13.1 = 7.9 + 5.2).

Scheme 2



$$\log K_{f,anion}^{H\ bond} - \log K_{f,acid}^{H\ bond} = pK_a^{int} - pK_a^{obsd}$$

$$pK_a^{int} \approx pK_a^{int\,\prime}$$



Results

The carboxylic acid pK_a's of compounds **1**, **2**, and **3** decrease as the number of hydrogen bonds increases (Table 1). Hydrogen bond formation and standard substituent effects can both alter the pK_a of the carboxylic acid. The analysis depicted in Scheme 2 was used to isolate the energetic effect of the hydrogen bond(s) (see also refs 2 and 3). The effect of the hydrogen bond(s) on these pK_a values can be estimated from the decrease in the experimentally observed pK_a (pK_a^{obsd}) relative to the "intrinsic" pK_a value of the compound (pK_a^{int}) (Scheme 2).[15] The

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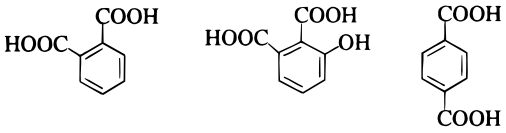
"intrinsic" pK_a refers to the pK_a value expected in the absence of hydrogen bonding (K_a^{int}, Scheme 2A). This was estimated as the pK_a of the corresponding compound with the hydroxyl group *para* to the carboxyl group instead of *ortho*, so that no intramolecular hydrogen bond can form (Scheme 2B and Experimental Section).[16]

The energetic effects of the hydrogen bonds estimated according to Scheme 2 are summarized in Table 1. The decrease in the pK_a of **2** by 5.8 units from the intrinsic value (6.6 vs 12.4) corresponds to stabilization of the salicylate monoanion, relative to the acid, by 7.9 kcal/mol from a single hydrogen bond. The 14.4-kcal/mol stabilization of the monoanion of **3** corresponds to an additional contribution of 6.5 kcal/mol from the second *o*-OH group.

In contrast, the monoanion of 3-hydroxyphthalic acid (**6**), in which an *o*-COOH group is present in addition to an *o*-OH group, is stabilized by 8.5 kcal/mol relative to the acid. This is only 0.6 kcal/mol more than the stabilization from the single *o*-OH group in the SA monoanion and is 4.6 kcal/mol less than an additive effect from the individual hydrogen bonds (Table 1).



5 **6** **7**

Discussion

The results with compounds **2** and **3** suggest that the energy of multiple hydrogen bonds can be large and nearly additive. Hydrogen bonding and other weak electrostatic interactions can be independent or nearly so, as each of these interactions, in contrast to covalent interactions, produces little change in charge distribution.[18] Thus, the combined energetic effects of multiple hydrogen bonding and other electrostatic interactions, even if individually modest, can be used by enzymes to achieve significant transition state stabilization. There is no requirement for a single interaction to provide most of the energy for catalysis.

Consistent with this view, enzyme active sites are typically replete with groups that interact with the substrate at positions undergoing charge rearrangement in the course of the reaction. For example, in the oxyanion hole of serine proteases, conserved backbone or side chain amides donate two hydrogen bonds to the carbonyl oxygen of the scissile amide of the substrate.[20] In the active site of mandelate racemase, one carboxylate oxygen of mandelate is hydrogen bonded to Lys164 and is further stabilized by an active site Mg^{2+} ion; the other carboxylate oxygen is hydrogen bonded to Glu317.[21] Similar patterns of multiple hydrogen bonding and metal coordination on the substrate at positions of charge rearrangement are observed for many other enzymes, including triose phosphate isomerase, enolase, ferricytochrome b_2, and citrate synthase.[22]

The observed near additivity of the energy of the two hydrogen bonds in **3** is consistent with pre-positioning of the hydrogen bond acceptor with respect to the hydrogen bond donors. This minimizes the entropic penalty that must be paid in forming the first hydrogen bond.[6,23] The energetic advantage of entropic fixation has been demonstrated in various model catalytic systems[24] and synthetic receptors.[8b] For **2** and **3** and

(15) This analysis assumes that the carboxyl and hydroxyl groups are hydrogen bonded in the acid species (Scheme 2A, lower left corner; note that other modes of hydrogen bonding between these groups are also possible). Intramolecular hydrogen bonding in the acid is expected to be favorable because DMSO is ineffective at stabilizing the lone pair electrons on the carbonyl oxygens[12] and the adjacently positioned *o*-hydroxyl group has both an electrostatic and entropic advantage in interacting with the carboxyl group. If intramolecular hydrogen bonding in the acid is not favorable, K_a^{obsd} represents the equilibrium between the non-hydrogen-bonded acid species and the hydrogen-bonded anionic species (Scheme 2, upper left and lower right corners). In such cases the analysis reduces to $\Delta G^{H\ bond} = -RT\ \ln K_{f,anion}^{H\ bond} = -RT\ \ln(K_a^{obsd}/K_a^{int})$, where $\Delta G^{H\ bond}$ represents the free energy of hydrogen bond formation in the monoanion and is a lower limit of the difference in free energy of the hydrogen bond in the anionic vs the acid species.

(16) In general, *ortho* and *para* substituents have similar resonance and polar effects on the acidity of benzoic acids.[17] Control experiments described below were carried out to determine if there are *ortho*-specific effects from steric or electrostatic features that might interfere with the analysis herein. The results indicate that such effects are likely to be small, changing the hydrogen bond energies by ≤ 1 kcal/mol and not affecting the conclusions concerning the additivity of the hydrogen bond energies. The similar pK_a values of 4-CH_3-BA, 2-CH_3-BA, and 2,6-$(CH_3)_2$-BA of 11.4, 11.2, and 10.8, respectively, suggest that steric effects from *o*-OH groups are expected to be small. Analogously, the similar pK_a values of *p*-methoxy, *o*-methoxy-, and 2,6-dimethoxybenzoic acids (pK_a's 11.8, 11.6, and 11.6, respectively), as well as the similar pK_a values of terephthalic acid (**7**) and phthalic acid monomethyl ester of 9.80 and 9.90,[2] respectively, suggest that the *para*-substituent constant provides a reasonable estimate for the intrinsic effect of an *ortho* substituent. An $\sim$1 pK_a unit larger electron-withdrawing effect for an *ortho* group than a *para* group was observed for F-, Cl-, and Br-substituted benzoic acids (pK_a's 10.0, 9.3, and 9.2 for the *o*-F, Cl, and Br compounds compared to the pK_a values of 11.0, 10.6, and 10.6 for the corresponding *para* compounds). A similar effect could be present for the OH substituent. Nevertheless, control experiments with 2,6-disubstituted benzoic acids (pK_a's 8.3 and 8.2 for the F and Cl substituent, respectively) indicated that the effects of the electron-withdrawing groups are additive, i.e., that a second *ortho* substituent has a similar effect as the first one. Thus, correcting the pK_a^{int} values for this apparent larger electron-withdrawing effect of *ortho* substituents does not change the conclusion that the energetic effects of hydrogen bonds are additive, though it lowers the estimated stabilization from each hydrogen bond by $\sim$1 kcal/mol. For example, if the pK_a^{int} value of SA is 1 unit lower than that estimated from the *para* compound, the pK_a^{int} value of 2,6-$(OH)_2$-BA would be correspondingly lowered by 2 units. This lowers the estimated stabilization from one and two hydrogen bonds to values of 6.5 and 11.7 kcal/mol, respectively. The corrected value of 11.7 kcal/mol remains similar to the value of 13.0 kcal/mol expected from an additive effect.

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(18) Bond distances, infrared spectra and other results provide strong evidence for some extent of covalent bond character for certain hydrogen bonds in nonaqueous environments.[10,19] However, the near additivity of the energy of hydrogen bonds observed in this study suggests that the electronic rearrangement that occurs upon formation of these hydrogen bonds is small. Additivity is expected for electrostatic interactions if each of these interactions produces little change in charge distribution. In contrast, an additive effect would not be expected for interactions that are predominantly covalent in character, as there is generally extensive electronic rearrangements upon formation of a covalent bond. This would weaken the ability of the group undergoing such a rearrangement to form a second covalent or noncovalent bond. Thus, these hydrogen bonds appear to be predominantly electrostatic in character.

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many other model systems, pre-organization is achieved by covalent bonds within the molecule. In enzymatic catalysis, binding energy from noncovalent interactions is typically used to arrange the active site in the folded protein and to bind and properly orient the substrate with respect to the catalytic groups.[6,23,25]

Proper prealignment of enzymatic functional groups with respect to the substrate groups undergoing charge rearrangement may also impose unfavorable interactions in the ground state (relative to solution), thereby maximizing the increase in interaction energy in going from the ground state to the transition state.[6,23] The following observations provide nonenzymatic examples in which geometrical constraints appear to weaken the hydrogen bond.

(a) The 2.7 kcal/mol weaker hydrogen bond in the phthalate monoanion (**5**) than that in the salicylate monoanion (**2**), despite the stronger hydrogen bond donating ability expected with the more acidic COOH in **5**,[2,3,26,27] suggests that the geometry in **5** is suboptimal for intramolecular hydrogen bonding.

(b) The 4.6 kcal/mol less than additive effect of the hydrogen bonds from the *o*-hydroxyl and carboxylic acid groups in **6** suggests that the hydrogen bonds are weakened. This probably arises from the repositioning that occurs upon addition of a second *ortho* substituent or upon formation of one or the other hydrogen bond.

For serine proteases, it has been suggested that geometrical constraints weaken the ground state hydrogen bonding between the amide groups in the oxyanion hole and the carbonyl oxygen of the substrate because the C═O bond is too short for optimal hydrogen bonding within the oxyanion hole.[6,20,23] In general though, the destabilizing constraints in model systems may be more pronounced than those imposed by an enzyme. Catalytic groups within enzymatic active sites are presumably not as rigidly positioned as groups that are covalently fixed in model systems. In addition, the geometrical changes in going from the ground state to the transition state are typically small compared to the geometrical differences between model compounds. The extent to which enzymes can take advantage of geometrical changes remains to be determined.

Conclusions

Use of multiple interactions by enzymes to provide specific transition state stabilization is a widely recognized principle. The system described herein provides a simple model for the ability of multiple pre-positioned hydrogen bonds to stabilize charges that develop in the course of a reaction. In contrast to the difficulty of isolating the contribution of individual interactions from the large networks of interactions at enzymatic active sites or in synthetic catalytic systems, the simplicity of this model has allowed the observed energetic effects to be dissected into contributions from individual hydrogen bonds. The results show that the energetic effects of multiple hydrogen bonds can be nearly additive, consistent with expectations for simple electrostatic effects.

These and previous results suggest several additional ways in which hydrogen bonds can contribute to enzymatic catalysis. Pre-positioning of hydrogen bonding groups in an enzymatic active site can optimize their ability to catalyze a reaction by minimizing the entropic penalty for hydrogen bond formation in the transition state.[6,23,25] Precise alignment of active site groups for transition state interactions may constrain the geometry for ground state interactions, thereby weakening ground state interactions relative to transition state interactions.[6,23] Finally, enzyme active sites can provide an environment in which there is a greater increase in hydrogen bond strength accompanying charge rearrangement than that in aqueous solution.[3,19,28,29] This difference is particularly pertinent as enzymes need to accelerate a reaction relative to that in water (see ref 3 and references therein for detailed discussions).

Experimental Section

pK_a Measurements. pK_a's in DMSO were measured by the overlapping indicator method[13,14] at 25 °C. The indicators used were 2,4-dinitrophenol (pK_a = 5.12), 2,6-di-*tert*-butyl-4-nitrophenol (pK_a = 7.3), and 9-carboxymethylfluorene (pK_a = 10.35), which were synthesized as described previously,[14] and bis(phenylsulfonyl)methane (pK_a = 12.25),[14] which was from Sigma. Multiple measurements were performed for each compound, using one or two indicators whose pK_a values are within two units from that of the compound being measured. The low pK_a's of 2,6-dihydroxybenzoic acid and 3-hydroxyphthalic acid were further confirmed by following the self-dissociation of the acid, as described previously.[14]

The effects of ion-pairing and acid self-association on the deprotonation of benzoic acids in DMSO have been extensively investigated in previous studies (refs 11 and 14 and references therein). These effects are small in general (e.g., $K_d^{dimer} \approx 0.02$ M for benzoic acid), and are even smaller for *ortho*-substituted benzoic acids such as **2**, **3**, **5**, and **6**.[11,13] The concentrations used herein (10^{-5}−10^{-3} M) were well below these dissociation constants. The following observations further suggest that complications from such effects are negligible: (1) Self-association gives deviations in plots of indicator absorbance vs concentration of the acid whose pK_a is being determined. Fits to the data were good ($R > 0.98$) without inclusion of a term for self-association, and including such a term gave no significant change in either the fits or the pK_a values obtained. (2) pK_a values obtained in titrations with different final acid concentrations (0.1 mM − 1 mM) were the same, within error (∼0.2 units). (3) The pK_a values obtained are similar when different counterions were used in the indicator method (potassium vs. tetraethylammonium), or when H^+ was used as the counterion in the self-dissociation method.

Estimation of pK_a^{int}. The measured pK_a of 12.4 (Table 1) for **4** was used as the pK_a^{int} value for salicylic acid (**2**). The intrinsic effect of the two hydroxyl groups of 2,6-dihydroxybenzoic acid was estimated by doubling the effect of a single *p*-hydroxyl group. That is, a *p*-hydroxyl substituent increases the pK_a of benzoic acid by 1.3 units, from 11.1 to 12.4; two hydroxyl substituents are then predicted to increase the pK_a by 2.6 units, to 13.7, which was then used as the pK_a^{int} value for **3**. The pK_a^{int} values of phthalic acid (**5**) and 3-hydroxyphthalic acid (**6**) were obtained analogously. The measured pK_a of 9.80 for terephthalic acid (**7**, Table 1) was used as the pK_a^{int} value of **5**. The intrinsic effects of a *p*-hydroxyl group, which increases the pK_a of benzoic acid by 1.3 units (cf. **1** vs **4**, Table 1), and a *p*-carboxylic acid group, which decreases the pK_a of benzoic acid by 1.3 units (cf. **1** vs **7**, Table 1), were combined to yield the estimated pK_a^{int} value of 11.1 for **6**.

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