Understanding enzymic catalysis: the importance of short, strong hydrogen bonds

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We have proposed previously that short, strong hydrogen bonds exist in enzyme active sites and that they are important in explaining enzymic rate enhancements. Here, we defend this proposal and provide evidence for likely changes of hydrogen bond strengths during enzymic catalysis.

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This article was invited in response to an article by Guthrie [Guthrie, J.P. (1996). Short strong hydrogen bonds: can they explain enzymic catalysis? *Chemistry & Biology* **3**, 163–170] that argued that short hydrogen bonds are not necessarily strong, and that there is therefore no evidence for strong hydrogen bonds in enzyme active sites.

Introduction

Previously, we proposed [1–4] that short, strong hydrogen bonds (also known as low-barrier hydrogen bonds or Speakman–Hadzi bonds) between reactive intermediates and active site proton donors are important in understanding the rates of many enzymic reactions. This was criticized recently by Guthrie [5] who claimed that the standard free energies of formation of analogous complexes in solution were not sufficiently negative. The critical issue is not, however, the standard free energy of formation of a hydrogen bond between unconnected partners, but whether a large increase in hydrogen bond strength can occur as the enzyme–substrate complex is converted to a reactive intermediate or transition state. Such an increase might be achieved by making the bonded bases more equal in strength, by compression, by extrusion of disordered water, by the introduction of supplementary hydrogen bonds, or by some combination of these factors.

A lively debate has emerged in the biochemical and chemical communities regarding the importance of short, strong hydrogen bonds in enzymic reactions. Guthrie focused on the standard free energies of formation of hydrogen bonds between separated reactants in solution. Scheiner [6], Herschlag [7] and others have focused on the importance of precisely matching the proton affinities of the hydrogen bond donors and acceptors, which was not part of our original proposal. In this article, we discuss hydrogen bonds in enzymes and their small molecule analogs in crystals and in solution and arrive at conclusions quite different from those of Guthrie.

Many enzyme-catalyzed reactions involve metastable intermediates for which the free energy of formation in solution, in the absence of the enzyme, is more positive than the free energy of activation of the enzyme-catalyzed reaction as assessed from k_{cat}, the first order rate constant with which the enzyme–substrate complex is converted to products. Such intermediates include enolate anions in racemization, isomerization, and Claisen condensation reactions of carbon acids as well as anionic tetrahedral intermediates in hydrolysis reactions of peptide bonds. The observed rates require that the enzymic transition states must be significantly stabilized relative to the analogous nonenzymic aqueous transition states.

Some authors ([8,9] and references therein) have proposed that multiple, small electrostatic interactions are primarily responsible for the differential stabilization of enzyme-bound reactive intermediates and transition

states. In contrast, we proposed that short, strong hydrogen bonds between bases of similar proton affinity could account for much of the required differential stabilization of many enzyme-bound intermediates and transition states compared to the initial enzyme–substrate complex [1–4]. Our proposal provided a simple explanation for the common observation that although the proton affinities of two basic sites, one in the enzyme active site and one in substrate, are quite different in the enzyme–substrate complex, they become more similar as the enzyme–intermediate complex is formed. We argue that this increase in similarity of basicity will increase the strength of the hydrogen bond significantly.

Guthrie [5] chose an arbitrary limit of $-10\,kcal\,mol^{-1}$ for the standard free energy of formation of a short, strong hydrogen bond from separated reactants in solution. When he failed to find examples that met his criterion, he concluded that "there is no evidence for strong hydrogen bonds in solution, and therefore that there is no basis for invoking such hydrogen bonds to explain enzymic catalysis" [5]. But short hydrogen bonds have been known for 48 years [10]. They are associated with very characteristic infrared (IR) [11,12] and nuclear magnetic resonance (NMR) [13] spectral features, which permit them to be identified unambiguously. The bridging hydrogen atom also has a characteristically small isotopic fractionation factor [13]. The spectral features and the energetics of short, strong hydrogen bonds are discussed in separate sections that follow.

When comparing a series of bonds between the same atoms, the shorter bonds are generally accepted as being the stronger [14,15]. But, the assignment of numerical values to individual bond strengths in polyatomic molecules is intrinsically ambiguous [16]. Furthermore, the quantity that Guthrie chose (free energy of formation) was not the best available model for the quantity of interest (the bond strength). Our assertions are supported and exemplified in the detailed sections that follow. We emphatically reject Guthrie's dismissal of short, strong hydrogen bonds as being irrelevant to enzymology.

In this review, we assert first that the experimental evidence available supports our original proposal that, as the enzyme–substrate complex is converted via a transition state to the enzyme–intermediate complex, hydrogen bond strengths vary more significantly than previously recognized; and second that differential stabilization of enzyme-bound intermediates and transition states by short, strong, low-barrier hydrogen bonds can, in fact, explain a significant fraction of the observed catalysis in a number of cases. We identify short, strong, low-barrier hydrogen bonds by means of their structural and spectroscopic characteristics and then discuss the energetic consequences of these bonds.

Short is strong

Although bond energies in solids are not easily estimated experimentally, the idea that hydrogen bonds become stronger as the donor–acceptor distance becomes shorter is generally accepted and leads to a rationalization of a large number of bond lengths [17]. In theoretical work, bond lengths emerge naturally and they have the anticipated relationship to energies of formation. Calculated energies of formation for substances B_2H^- from B^- and BH, where B^- is a variety of anionic carbon bases, range from $2.3\,kcal\,mol^{-1}$ to $19.5\,kcal\,mol^{-1}$, with a corresponding monotonic decrease in donor–acceptor distance from 403 pm to 320 pm [18]. (In this work, the same basis set, MP2/6–31+G**, was used in all but one of the calculations and the nonconforming basis set was similar. In the other comparisons, the same basis sets were used throughout the comparison.) Hydrogen bond energies for various spectroscopic states of enol-malondialdehyde vary from $0.5\,kcal\,mol^{-1}$ to $23.6\,kcal\,mol^{-1}$, with a corresponding monotonic variation of the oxygen–oxygen distance from 296.2 pm to 256.6 pm [18]. Energies for hydrogen bonding ammonia to various protonated amines range from $23.9\,kcal\,mol^{-1}$ to $29.9\,kcal\,mol^{-1}$ (with the strongest bond to NH_4^+). The corresponding, monotonic variation in nitrogen–nitrogen distance is from 281.7 pm to 273.1 pm [19]. Energies for hydrogen bonding H_2O with several protonated oxygen bases range from $32.0\,kcal\,mol^{-1}$ to $43.9\,kcal\,mol^{-1}$ (with the strongest bond to H_3O^+). The corresponding, monotonic variation in oxygen–oxygen distances is from 248.3 pm to 237.0 pm [19]. In a very recent theoretical study [20] a 241 pm donor–acceptor distance was found in the maleate monoanion, compared with 265 pm in maleic acid (also hydrogen bonded) and 264 pm in maleic acid monoamide. The calculated *cis* to *trans* isomerization free energy of the maleate monoanion was $7\,kcal\,mol^{-1}$ more positive than that of the monoamide and $20\,kcal\,mol^{-1}$ more positive than that of the diacid [20].

The principle that shorter is stronger seems quite general, although there may be occasional exceptions where the geometry is distorted by other structural features. How strong remains to be estimated in particular cases.

Structure and spectra in various environments

A large body of experimental evidence is available describing the properties of short, strong hydrogen bonds between two oxygen atoms in small molecules both in the solid phase and in solution. In many such crystals the hydrogen bond is bisected by one of the symmetry elements of the unit cell, but exact symmetry is not required for the formation of a short, strong hydrogen bond [21,22]. The O···O distances in such compounds are typically ≤ 2.5 Å [23,24]. Hydrogen bonds between bases of dissimilar proton affinities are considerably longer.

Short, strong, low-barrier hydrogen bonds produce characteristic spectral properties. Their IR spectra contain no

hydroxyl absorption band in the usual 3000–2000 cm^{-1} region; instead, the spectra contain a very broad and intense band between 2000 cm^{-1} and 500 cm^{-1}, with the width-at-half-height >500 cm^{-1}. Such spectra have been designated Hadzi type ii spectra [11,12]. The hydrogen bonded protons in these compounds also have unusual downfield chemical shifts in their ^1H NMR spectra, usually >15 ppm downfield from tetramethylsilane (TMS) [13]. For example, in the solid phase hydrogen bis(4-nitrophenoxide) anion has a Hadzi type ii IR spectrum [25]. When its hydrogen-bonded proton is replaced with ^2H the chemical shift is 16.4 ppm [26]. Importantly, these properties persist when such substances are dissolved in nonhydroxylic solvents [27]. The ^1H NMR chemical shift of the bridging proton of the bis(4-nitrophenoxide) ion in acetonitrile is 16.9 ppm [13]. The IR spectrum of tetrabutylammonium hydrogen bis(4-nitrophenoxide) in acetonitrile is the same as in the crystalline solid (S.S. Marimanikkuppam and M.M.K., unpublished observation). When the bridging ^1H in a short, strong hydrogen bond is replaced with ^2H, the relevant NMR resonance is shifted upfield by ~0.6 ppm; if ^3H is used, a further upfield shift occurs [28]. These shifts are observed in solution. Thus, short, strong hydrogen bonds can and do exist in nonhydroxylic solvents.

Short, strong hydrogen bonds can also coexist with water in an ordered crystalline environment. For example, the relevant O···O distance in sodium hydrogen bis(4-nitrophenoxide) dihydrate is 2.465 Å; in two unhydrated salts, the distances are 2.475 Å and 2.48 Å [26]. Many of the short, strong hydrogen bonds reported earlier occur in hydrated crystals [23,24]. Short, strong hydrogen bonds have also been observed in concentrated aqueous solutions of strong acids [29]; they were identified by their Hadzi type ii IR spectra. Although water is both a hydrogen bond donor and a hydrogen bond acceptor, ordered water molecules need not disrupt short, strong hydrogen bonds [26]. We agree with Guthrie [5] that short, strong hydrogen bonds probably do not occur in dilute aqueous solution. But, in concentrated aqueous solution almost all the water is organized to solvate the solutes, and short, strong hydrogen bonds are possible.

Free energy versus enthalpy

Guthrie discussed standard free energies of formation of hydrogen bonded substances [5]. With the exception of hydrogen bonding in monohydrogen phthalate, his examples were bimolecular processes that require the donor and acceptor to be localized and oriented in order to form the hydrogen bond. The free energy changes (–6 kcal mol^{-1} to –7 kcal mol^{-1}) cited by Guthrie are influenced by probability (entropy) changes as well as energy (enthalpy) changes; the entropic contribution makes the formation of the hydrogen bonded species less favorable because the number of available arrangements of the system is reduced. The amount of this entropic contribution is a function of the standard state that is defined. The processes we are trying to understand, the conversion of enzyme–substrate complexes to active intermediates or transitions states, are unimolecular and therefore do not have the problem of an unfavorable entropic contribution. Conventional covalent bond strengths are enthalpies, not free energies. Where they are available, the standard enthalpies of formation of short, strong hydrogen bonds are in the range –10 kcal mol^{-1} to –15 kcal mol^{-1} [30,31] (i.e. 3–8 kcal mol^{-1} more negative than the standard free energies). We recognize, however, that the standard enthalpies probably contain contributions from electrorestriction of the solvent, and thus they are probably more negative than the quantities we seek. The answers most relevant to enzymology probably lie somewhere between ΔH^0 and ΔG^0.

If standard free energies of bimolecular processes are to be used as a model for the free energy associated with the conversion of the enzyme–substrate complex to the enzyme transition state complex, they should be corrected by at least –2 kcal mol^{-1}, assuming a 1 M standard state. This is a minimum correction, principally based on the loss of entropy on mixing one species with the solvent [32]. The real correction should also take account of the orientational constraints imposed by the hydrogen bond and would certainly be more negative, but it is hard to estimate these further corrections. Using –2 kcal mol^{-1} as a correction, the enthalpies of formation of intermolecular hydrogen bonds for the examples cited by Guthrie are –8 kcal mol^{-1} to –9 kcal mol^{-1} in dimethylsulfoxide (DMSO). More realistically, slightly larger correction values would push many of these above 10 kcal mol^{-1}, into the range of the hydrogen bonding enthalpies cited above. This is sufficient to have a very powerful impact on the rates of enzyme-catalyzed reactions. But we believe that the structural and spectroscopic criteria are much more appropriate indicators of the existence of short, strong, low-barrier hydrogen bonds than the free energy of formation. The standard free energy changes associated with intramolecular hydrogen bond formation in salts of monohydrogen phthalate (as well as of monohydrogen maleate [33]) are not subject to these considerations. Structural and spectroscopic results [34–36] suggest, however, that these salts may not be good examples of short, strong hydrogen bonds.

The relevance of proton sponges

In our original work [3,4], we compared the basicity of proton sponges with that of 1-dimethylaminonaphthalene and estimated the hydrogen bond energy in the protonated sponge to be 17 kcal mol^{-1}. This was the origin of our statements that the energy of short, strong hydrogen bonds could reach 10–20 kcal mol^{-1} [6–9]. Guthrie [5] argued that repulsive interactions are responsible for the remarkably high pK_a values of the protonated sponges.

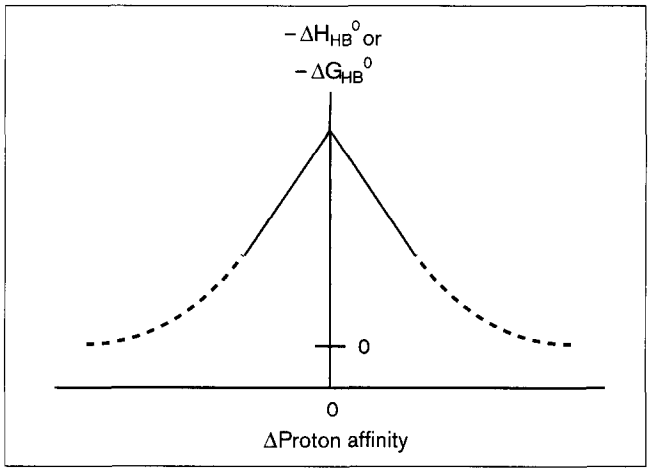
We do not disagree, but bond energy is simply the enthalpy of the system without the bond in question minus the enthalpy with it [16] and we believe that the proton affinity of the sponges is instructive for understanding enzymic catalysis. The formation of the enzyme–substrate complex may cause an interpenetration of nonbonded van der Waals spheres. The resulting positive contributions to the enthalpy would be coupled to the larger negative contributions from binding. If in the transition state this became the site of a hydrogen bond between bases of roughly equal proton affinity, the activation energy would be substantially reduced. In very favorable cases this reduction may be comparable to the hydrogen bond energy of the proton sponges. Although we know of no such cases at present, the value serves as a useful marker for the upper limit of plausible effects.

The conversion of substrate to a reactive intermediate

The foregoing analysis and other recent results [37] indicate that intramolecular hydrogen bond strengths can exceed 10 kcal mol^{-1} when the proton affinities of the donor and acceptor are very similar, as they can be in an enzyme–intermediate complex or transition state. If the hydrogen bond strength is significantly less when the proton affinities of the donor (DH) and acceptor (A^-) are dissimilar (i.e. in the enzyme–substrate complex) the energetic cost of forming the active intermediate or transition state should be substantially reduced. Even energies in the range 6–10 kcal mol^{-1} correspond to very large effects on rates and equilibrium constants. The key relationship is the dependence of hydrogen bond strength on the change in relative proton affinities.

Within structurally homogeneous families of compounds, the standard free energy, as well as the enthalpy, for the formation of a hydrogen bonded complex becomes steadily more negative as ΔpK_a becomes less positive or more negative. The localization (entropic) problem does not occur because the reactions are all of the same molecularity within a family. This is true both in solution and in the gas phase. In cases where DH and A^- are both part of the same molecule a similar conclusion can be inferred about the formation of hydrogen bonded from non-hydrogen-bonded structures. If we assume ΔpK_a to be proportional to the difference in proton affinities between A^-, and DH within the hydrogen bonded complex, we may expect that the complex will resemble DH···A^- when ΔpK_a is large and positive, D^-···HA when ΔpK_a is large and negative, and something intermediate when ΔpK_a is near zero. Because hydrogen bond energies are usually defined by comparing the energy of the hydrogen bonded substance with that of the more stable of the pairs, DH + A^- or D^- + HA, within a structurally homogeneous family, the hydrogen bond energy reaches a maximum when the proton affinities of D and A are equal. The derivative of

Figure 1



The expected dependence of $\Delta H_{HB}{}^0$ or $\Delta G_{HB}{}^0$ on the difference of the proton affinity between the donor and acceptor atoms (Δproton affinity). The solid lines indicate those values of Δproton affinity for which the Brønsted relation described in the text is valid [7,36–40]. The dotted lines describe the prediction that as the absolute value of Δproton affinity increases, the Brønsted β will decrease and approach zero.

hydrogen bond energy with respect to ΔpK_a may approach 0.5/RT near $\Delta pK_a = 0$ if both are measured under the same conditions. In the limit of large positive or large negative ΔpK_a the hydrogen bond energy goes to zero, so its derivative with respect to ΔpK_a must also go to zero. Figure 1 shows the likely dependence of $\Delta H_{HB}{}^0$ (or log K_{HB}) on the difference of the proton affinity between the donor and acceptor atoms. The standard free energy of formation, $\Delta G_{HB}{}^0$, would behave similarly. This analysis is consistent with the existence of a Marcus-like relation between hydrogen bond energy and ΔpK_a, as suggested by Magnoli and Murdoch [38]. Experimentally, such plots appear linear because it is very hard to extend ΔpK_a sufficiently to observe the curvature within a structurally homogeneous family. This is also true of the kinetic analog [39].

The apparent linearity of Brønsted-like plots [7,37,40–45], represented by:

$$\log K_{HB} = \beta \times \Delta pK_a + \text{constant},$$

was used by Shan and Herschlag [40] to estimate the amount by which the formation of a strong hydrogen bond may reduce the energy required to form an enzymic transition state from an enzyme–substrate complex. Because of the importance of this analysis, we describe the details for enolization reactions.

The carbonyl/carboxyl compounds that are the substrates in enzymic enolization reactions have pK_a values in the

range –2 to –8 in aqueous solution; their enol tautomers have pK_a values in the range 7–12 in aqueous solution. Thus, the ΔpK_a value accompanying enolization is ~15. Aqueous solution values of ΔpK_a can be used in applying the Brønsted relation if comparisons are limited to well defined, homologous systems [42,43].

The value of the Brønsted value β is solvent dependent, with the value much larger in nonhydroxylic solvents than in water [3,37,40,45]: 0.05–0.17 for water (dielectric constant 78.5), 0.7–0.9 for DMSO (dielectric constant 43), and 0.63–1.0 for tetrahydrofuran (THF; dielectric constant 7). Assuming that disordered water is not abundant near the active site of enzymic transition states and high energy intermediates, we have assigned β a value of 0.7. This value predicts an increase of $10^{10.5}$ in K_{HB} for the enolization reactions. This increase would result in a corresponding stabilization of the transition state by 14 kcal mol^{-1} at 25°C, and the rate constant for conversion of the enzyme–substrate complex to the enzyme–intermediate complex would increase by the same factor, $10^{10.5}$. This calculation is likely to overestimate the effect, however, because it is unlikely that the Brønsted relation, given above, applies to such a large change in structure and pK_a (as shown in Fig. 1). But this calculation does give a valid indication that the effects on the rate constants for enzyme-catalyzed reactions can be quite large.

Further differential stabilization of the enzymic transition state and activated intermediate can be achieved by compression, as in the proton sponges, and manipulation of water molecules in the catalytic site. The hydrogen bonds of the enzyme–substrate complex may be attenuated by the presence of disordered water [46]. This disordered water can be frozen into a bond-strengthening configuration, or extruded, in forming a transition state or activated intermediate [47]. We believe that many enzymic transition states contain little or no disordered water in the near neighborhood of their active sites. These factors further increase the possible effect of a short, strong hydrogen bond on the rate constant.

Why are short hydrogen bonds strong?

It is not necessary to know the origin of the binding energy to estimate the effect of a short, strong, low-barrier hydrogen bond on a catalytic rate constant. But we believe that they have a significant degree of covalent character and that this makes it easier to understand their properties. Space does not permit a full discussion, but we call attention to several salient points.

Centralization of the hydrogen

When ΔpK_a is zero, the proton is equally attracted to D^- and A^-. The substance may then have a centralized proton or be an equal mixture of DH···A^- and D^-···HA. A considerable number of substances in which the proton affinities of A^- and D^- have been equalized by making them the same has been carefully studied by neutron diffraction. The usual outcome is that the D–H and A–H bonds are significantly lengthened, but the proton appears to be distributed between the two sites [23]. At least one substance is known, however, in which the bridging proton is centralized [23]. Here, both of the O–H bonds in the bridge have covalent character. We believe it is unlikely that structural chemistry contains discontinuities. Since one substance exists in which both parts of the hydrogen bond clearly have covalent character, the same is probably true of structurally similar substances. The lengthening of the O–H bond in other substances with short, strong hydrogen bonds tends to confirm this conclusion.

Electron redistribution

Crystals of potassium hydrogen acetylenedicarboxylate contain intermolecular short, strong hydrogen bonds with an O···O distance of 2.445 Å. In the crystal the intensity of the asymmetric C=O stretching band of potassium hydrogen acetylenedicarboxylate is greatly increased by hydrostatic pressure, with a pressure of 6 GPa increasing the intensity of the band by more than fivefold [48]. Substitution of ^2H for ^1H in short, strong hydrogen bonds increases the O···O distance [20], and in potassium hydrogen acetylenedicarboxylate the intensity of the C=O asymmetric stretching band is decreased by substituting ^2H for ^1H in the hydrogen bonding position. These changes in intensity demonstrate that shortening or lengthening the hydrogen bond results in a redistribution of electron density in the carboxyl group and provide direct experimental evidence for electron redistribution (charge transfer, a nonelectrostatic effect [48]) in the formation of short, strong hydrogen bonds.

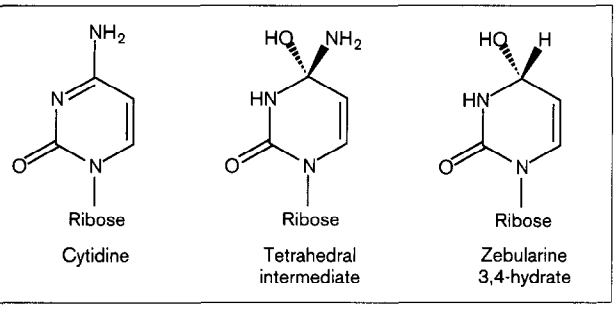
Finally, we note that ~2 kcal mol^{-1} of the binding energy of short, strong hydrogen bonds is the delocalization energy of the proton [26,49] (i.e. the greatly reduced zero-point energy of the hydrogenic vibrations). This effect is also the main cause of the low isotopic fraction factors to which we have alluded [13].

When the van der Waals radii of interacting atoms overlap, the decomposition of the bond energy into electrostatic and covalent parts is necessarily somewhat arbitrary [50]. In short, strong hydrogen bonds the van der Waals radii of the donor and acceptor atoms overlap, and the hydrogen is almost completely overlapped by both. Thus, it is clear that no unique decomposition is possible. Nevertheless, it seems to us that short, strong hydrogen bonds have important nonelectrostatic contributions.

Short, strong hydrogen bonds in active sites

The existence of short, strong hydrogen bonds between stable inhibitors and active site electrophilic groups has been revealed by X-ray crystallography and ^1H NMR

Figure 2



The structures of cytidine, the tetrahedral intermediate of cytidine and zebularine 3,4-hydrate, which mimics the tetrahedral intermediate.

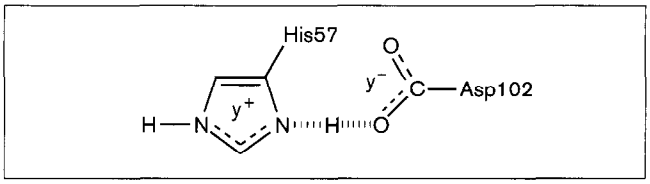
spectroscopy. In some cases, kinetic data were used to estimate the strengths of these hydrogen bonds. These values are sufficient to contribute significantly to the rates of the enzyme-catalyzed reactions.

The X-ray structure of cytidine deaminase complexed with zebularine 3,4-hydrate (see Fig. 2), a mimic of a tetrahedral intermediate and a competitive inhibitor with a $K_i = 1.2 \times 10^{-12}$ M, reveals a short hydrogen bond between the OH group of the pyrimidine ring and the carboxylate group of Glu104 (2.45 Å) [51]. This distance is ~0.5 Å less than the van der Waals contact distance [52] and the hydrogen atom is completely overlapped. Because this OH group is coordinated to an active site Zn(II), its pK_a is reduced towards the value of the carboxylic group of Glu104. In the Glu104→Ala mutant, k_{cat} is reduced by a factor of 10^8, corresponding to an increase in the activation energy by 11 kcal mol^{-1} [53]. This decrease in rate reflects the energetic importance of the short, strong hydrogen bond in catalysis as well as the use of the carboxylate group in transferring protons.

Both ^{1}H NMR and X-ray crystallography have been used to examine the hydrogen-bonded proton shared by Asp102 and His57 (nominally, protonated His57) in α-chymotrypsin (Fig. 3). At pH 3.5 and in the absence of an inhibitor, Frey *et al.* [4] observed that in H_2O the ^{1}H NMR signal for this proton occurs at a chemical shift of 18.3 ppm and in D_2O the ^{2}H NMR chemical shift of the hydrogen bonded deuteron is shifted upfield by 1.0±0.4 ppm. Both characteristics were in agreement with the behavior expected for short, strong hydrogen bonds [13].

Abeles, Ringe and coworkers [54,55] studied the tetrahedral adducts formed when peptidylfluoromethylketones were complexed with Ser195 (Fig. 4). The ^{1}H NMR chemical shift of the proton shared by Asp102 and His57 was reported to be 18.7 ppm, and the pK_a of His57 was reported to be > 10.5 [54]; more recent studies found that the chemical shift is 18.9 ppm, and the pK_a of His57 is

Figure 3



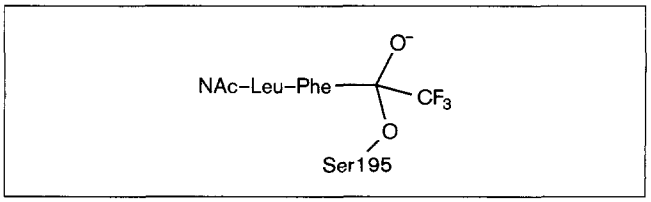
An illustration of the hydrogen bond between Asp102 and His57 in α-chymotrypsin.

≥12 [56]. The latter pK_a value is elevated by ≥5 units from the value for histidine in solution, indicating that the short, strong hydrogen bond is > 7 kcal mol^{-1} stronger than a conventional imidazolium hydrogen bond in water. The N···H···O distance in this complex is 2.52 Å [55], 0.55 Å less than the van der Waals contact separation [52], and the hydrogen is completely overlapped. This hydrogen bond satisfies the criteria for a short, strong hydrogen bond and has sufficient strength to contribute significantly to the catalytic rate by stabilizing the enzyme-bound tetrahedral intermediate.

The conclusion that protons shared between Asp and His can form strong bonds are supported by the geometry of the hydrogen bond in a crystal of a model of the Asp–His dyad. Gandour *et al.* [57] studied a compound in which an intramolecular hydrogen bond could form using the *anti* orbital of the benzoic acid group and the benzimidazole. The crystal structure, however, revealed an intermolecular hydrogen bond that employed the more basic *syn* orbital of the carboxylic acid group (Fig. 5) [58]. The length of this hydrogen bond, 2.59 Å, is less than the van der Waals contact separation [52], so the hydrogen is completely overlapped (i.e. the hydrogen bond is a short, strong hydrogen bond and the pK_a values of the carboxylic acid and the conjugate acid of the benzimidazole are nearly equivalent).

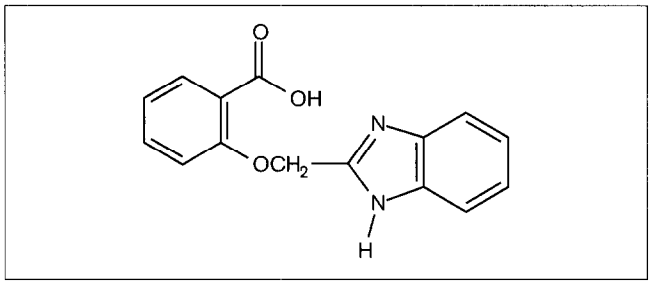
Mildvan and coworkers [59] studied the interaction of the competitive inhibitor dihydroequilenin (Fig. 6) with ketosteroid isomerase; dihydroequilenin is a mimic of the

Figure 4



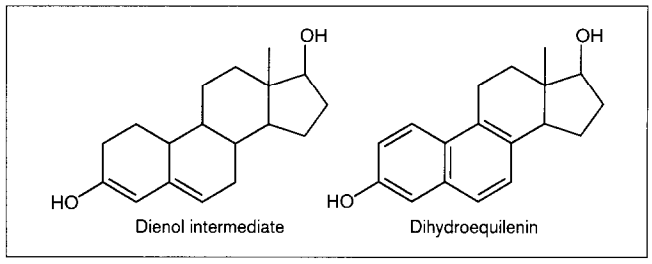
An example of the tetrahedral adducts formed when peptidylfluoromethylketones complex with Ser195 in α-chymotrypsin.

Figure 5



The compound used by Gandour *et al.* [57] to model the Asp–His dyad. In this compound, an intermolecular carboxyl–imidazole hydrogen bond forms using the *syn* orbital of the benzoic acid group. A nearly centrally located proton results.

Figure 6



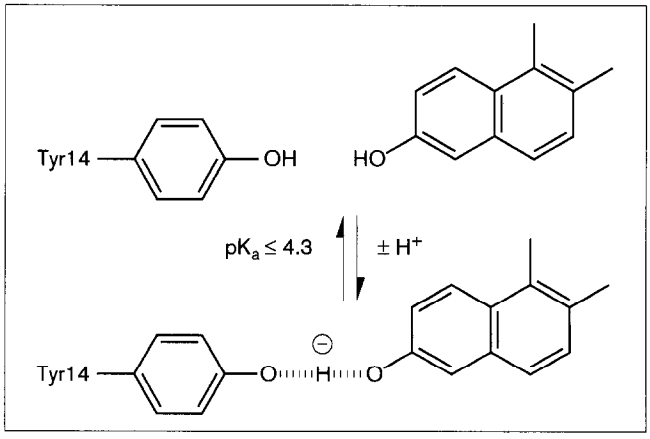
The structures of the dienol intermediate in the 1,3-proton transfer reaction [59] and its mimic, dihydroequilenin.

dienolic intermediate (Fig. 6) in the 1,3-proton transfer reaction. The reaction is facilitated by Tyr14, an electrophilic catalyst, and Asp38, a general base catalyst. The pK_a values of dihydroequilenin and Tyr14 are closely matched [59]. In the inhibited complex the ^1H NMR chemical shift of the proton shared by Tyr14 and the inhibitor is 18.15 ppm, and the fractionation factor is 0.34; these are characteristic for short, strong hydrogen bonds [13]. The rates of dissociation of the inhibitor from the wild-type isomerase and the Tyr14→Phe mutant were also measured, and the strength of the hydrogen bond in the wild-type enzyme was estimated to be $\geq 7.6\,kcal\,mol^{-1}$. This value is similar to that deduced ($\geq 7.8\,kcal\,mol^{-1}$) from the pK_a value (≤ 4.3) for the proton loss that occurs on formation of the isomerase–dihydroequilenin complex (Fig. 7).

As noted by Guthrie [5], a short hydrogen bond has been observed by X-ray crystallography in the active site of an inhibited citrate synthase [60]. The carboxylate group of the competitive inhibitor *S*-carboxymethyl CoA, a carboxyl analog of acetyl CoA, is hydrogen bonded to the carboxylate group of Asp375, the putative general base catalyst in the enolization of acetyl CoA. The O···O distance in a 2.0 Å resolution structure is reported to be < 2.4 Å. In contrast, the O···N distance for the complex with an analogous amide containing inhibitor is ~2.5 Å. Assuming that the accuracies of these structures allow the interpretation of the hydrogen bond lengths, Drueckhammer and coworkers [60] proposed that a short, strong hydrogen bond is formed with the carboxyl inhibitor while a normal hydrogen bond is formed with the carboxamide inhibitor. But, measurements of the K_i values for these inhibitors revealed a difference of only a factor of 18 ($2\,kcal\,mol^{-1}$), although the aqueous solution pK_a values of carboxylic acids and carboxamides differ by ~13 units. The K_i value for the carboxyl inhibitor was demonstrated to be pH dependent, as expected if a proton is required to form the bridge. The lowest pH value used in these experiments, pH 6.0, exceeds the expected pK_a of either Asp375 or the inhibitor by ~1–2 units. This leads to an underestimate of the effect of strengthening the hydrogen bond, probably by a factor of ~10. Thus, this short hydrogen bond appears to contribute $\sim 3\,kcal\,mol^{-1}$ to inhibitor binding, beyond the contribution of the 'normal' hydrogen bond.

Drueckhammer and coworkers [60] noted, however, that this short, strong hydrogen bond is formed with a *syn* orbital of the carboxylate group of Asp375 and an *anti* orbital of the carboxylate group of the inhibitor. Because the *syn* orbital of a carboxylate group has a higher proton affinity than the *anti* orbitals (a factor of $\sim 10^4$; [57]), the Brønsted relation predicts that the strength of this hydrogen bond will not be maximal. Using 0.7 as the value for the Brønsted β, the strength of this hydrogen bond is predicted to be $\sim 4\,kcal\,mol^{-1}$ less than the maximal value when the pK_a values are equal (i.e. a short, strong hydrogen bond between two carboxylic acid/carboxylate groups could provide $7\,kcal\,mol^{-1}$ of differential stabilization to transition states and intermediates in an enzyme active site). The reaction catalyzed by citrate synthase does not,

Figure 7



The formation of a hydrogen bond between the isomerase and dihydroequilenin. The corresponding hydrogen bond strength is $\geq 7.8\,kcal\,mol^{-1}$.

however, involve such a hydrogen bond since Asp375 is the general base catalyst in the enolization of acetyl CoA.

Guthrie [5] also questioned the importance of short, strong hydrogen bonds in the reactions catalyzed by other enzymes. Kollman and coworkers [61] calculated that proton transfer from His95 to an enediolate intermediate in the active site of triose phosphate isomerase was unfavorable, thereby making it unlikely that a short, strong hydrogen bond could be formed that would stabilize the intermediate. Instead, they proposed that electrostatic interactions involving His95 and Lys12 stabilize the intermediate. This calculation neglected the ability of the active site environment to stabilize the imidazolate anion. Recent calculations performed by Guo and Salahub [62] revealed the importance of a hydrogen bond between His95 and the NH of the peptide bond between Ser96 and Glu97 in stabilizing the imidazolate anion. The proton affinities of an imidazolate anion and the conjugate base of a peptide bond are similar, thereby providing stabilization via the formation of a short, strong hydrogen bond.

In the active site of ribonuclease A, the ε-ammonium group of Lys41 has long been assigned a role in stabilizing a pentacoordinate transition state or intermediate. By site-directed mutagenesis and chemical modification, Raines and coworkers [63] substituted Lys41 with a variety of neutral and cationic groups. While k_{cat} was observed to be remarkably insensitive to the position of the cationic charge of residue 41, the substitution of a trimethylammonium group for the ammonium group inactivated the enzyme. This suggests that the functional group of residue 41 is required to participate in a hydrogen bond rather than only interact electrostatically with the intermediate/transition state, as previously suggested by Gerlt and Gassman [2].

Conclusions

Short, strong hydrogen bonds exist in solution. Based upon the properties of low molecular weight models, the changes in the hydrogen bond strengths that are likely to occur as an enzyme–substrate complex is transformed to an enzyme–intermediate complex and/or transition state can be of sufficient magnitude to contribute very significantly to enzyme catalysis. The available enzymological data are consistent with the presence and participation of short, strong hydrogen bonds in enzyme–catalyzed reactions. While the magnitudes of their energetic contributions to catalysis needs further investigation, the existence of short, strong hydrogen bonds in enzyme active sites appears certain.

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