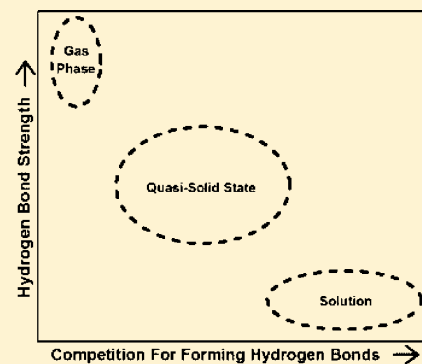


Strong, Low-Barrier Hydrogen Bonds May Be Available to Enzymes

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ABSTRACT: The debate over the possible role of strong, low-barrier hydrogen bonds in stabilizing reaction intermediates at enzyme active sites has taken place in the absence of an awareness of the upper limits to the strengths of low-barrier hydrogen bonds involving amino acid side chains. Hydrogen bonds exhibit their maximal strengths in isolation, i.e., in the gas phase. In this work, we measured the ionic hydrogen bond strengths of three enzymatically relevant model systems in the gas phase using anion photoelectron spectroscopy; we calibrated these against the hydrogen bond strength of HF_2^- , measured using the same technique, and we compared our results with other gas-phase experimental data. The model systems studied here, the formate–formic acid, acetate–acetic acid, and imidazole–imidazole anionic complexes, all exhibit very strong hydrogen bonds, whose strengths compare favorably with that of the hydrogen bifluoride anion, the strongest known hydrogen bond. The hydrogen bond strengths of these gas-phase complexes are stronger than those typically estimated as being required to stabilize enzymatic intermediates. If there were to be enzyme active site environments that can facilitate the retention of a significant fraction of the strengths of these isolated (gas-phase), hydrogen bonded couples, then low-barrier hydrogen bonding interactions might well play important roles in enzymatic catalysis.



Enzymes are remarkably efficient catalysts, notable for causing rate enhancements of up to 10^{26} -fold with great specificity under gentle conditions.¹ In the early days of enzymology, it was frequently assumed that there must exist some unknown physicochemical phenomenon that was making a large contribution to these impressive rate enhancements. Even now, 50 years since modern methods were first brought to bear and with enzymatic catalysis firmly established as a pillar of biochemistry, the basis for the proficiency of enzymes, i.e., their “secret”, remains elusive.

The early 1990s saw a flurry of activity that provided clues for elucidating this issue. In 1991, the X-ray structure determination by Petsko and Ringe² of a triosephosphate isomerase–transition state analogue complex³ and the simultaneous nuclear magnetic resonance (NMR) and infrared work of Knowles^{4,5} showed that neutral His-95 is the general acid stabilizing the enediolate intermediate in the reaction catalyzed by triosephosphate isomerase (TIM). In 1993, Gerlt and Gassman^{6,7} estimated that TIM His-95 was stabilizing the enediolate intermediate by at least 7 kcal/mol, and they postulated that this occurred because the imidazole side chain of neutral His-95 and the enediolate intermediate had matching pK_a values, facilitating the formation of a short, strong (ionic) hydrogen bond between them. At about the same time, Cleland and Kreevoy⁸ as well as Frey⁹ also postulated the formation of strong, low-barrier hydrogen bonds (LBHBs) between moieties with matching pK_a values to be an integral part of enzyme catalytic mechanisms. Over time, additional circumstantial evidence came to light in support of the LBHB hypothesis.^{10,11} Often, LBHBs were recognized in enzyme structures by their short lengths, their small deuterium

fractionation factors, and/or their large downfield, proton NMR chemical shifts.^{12,13}

While the LBHB hypothesis in enzyme catalysis had its proponents, it also had its critics, and it remains a controversial issue to this day.^{14–16} Its opponents note that the existence of ionic hydrogen bonds in enzyme active sites does not in itself imply that they are unusually strong there, and in fact, the opponents doubt that they are. Arguments from both sides are often based on the known or presumed strengths of ionic hydrogen bonds in different environments. Everyone agrees that hydrogen bonds are at their strongest in the gas phase, i.e., *in vacuo*. Moreover, species whose hydrogen bonds are strong in the gas phase often exhibit quite weak hydrogen bonding in aqueous solution, this likely being due to competition with water for hydrogen bonding. Both Guthrie and Perrin cite the weakening of hydrogen bond strengths in water as evidence that proponents’ arguments based on strong hydrogen bonds in the gas phase are not relevant to hydrogen bond strengths in enzymes.^{14,15} In solids, X-ray crystallography has provided many examples of short hydrogen bonds in enzyme structures. Nevertheless, these structural findings are unconvincing to some critics; they do not concede that short hydrogen bond lengths in crystalline enzyme structures imply strong hydrogen bonds.¹⁶ They furthermore argue that hydrogen bonding in crystals is simply not relevant to the environment of the enzyme’s active site.

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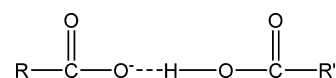
Trending against these criticisms of the LBHB hypothesis are two observations about the cloistered environments of enzyme active sites, on which the viability of short, strong hydrogen bonds there critically depends. First, enzyme active sites typically possess protein loops that fold down over the bound substrate to exclude water.¹⁷ Thus, these sites are largely sequestered from water, making the fact that hydrogen bonds are weakened in aqueous solution beside the point; enzyme sites are not typical aqueous environments. Second, the expectation of strong enzyme–transition state interactions, with the enzyme “bear-hugging” the transition state,¹⁸ suggests a relatively compact, semirigid environment at the enzyme active site and brings to mind the relatively immobile, structured environments of crystals, where there is evidence of short, possibly strong hydrogen bonds in some enzyme structures. Both of these characteristics are enabled by the macromolecular architecture of enzymes.

An essential tenet of the criticism against the LBHB hypothesis is that the strengths of enzymatically relevant hydrogen bonds would have to be unexpectedly high for the hypothesis to be plausible. Indeed, under the LBHB hypothesis, hydrogen bond strengths in the range of 10–20 kcal/mol have been proposed as being necessary to account for the stabilization of enzymatic intermediates.^{8,19} How might nature achieve such high hydrogen bond strengths at enzyme active sites? Hydrogen-bonded couples exhibit their optimal geometric structures and thus their maximal strengths in the gas phase, i.e., *in vacuo*, primarily because there they do not have competing hydrogen bonding interactions. In condensed-phase environments, where potentially competing hydrogen bonding interactions among molecules are plentiful and the optimal structures of hydrogen-bonded couples are correspondingly compromised, the average hydrogen bond strength is substantially weaker than in its gas-phase counterpart. Environments that suppress competition for forming hydrogen bonds might be expected to allow hydrogen-bonded couples to retain a portion of their *in vacuo*, hydrogen bond strengths. The nearly water-free, quasi-rigid structures of enzyme active sites are potentially opportune environments. Thus, it is plausible that some enzyme active sites may provide favorable environments in which hydrogen bond strengths retain a substantial fraction of their gas-phase strengths.

Because hydrogen bonds are at their strongest in the gas phase, the strength of a given hydrogen bond there provides an upper limit to its maximal strength in any other environment. In effect, the strength of a hydrogen-bonded couple in the gas phase tells us what would be possible in an optimized environment. Thus, measurements of hydrogen bond strengths in the gas phase supply upper limits to their strengths, providing important boundaries. Setting a quantitative benchmark for how strong hydrogen bonds can be at their strongest is the hydrogen bifluoride anion, HF_2^- , in the gas phase. This hydrogen-bonded pair, i.e., $\text{F}^- \cdots \text{HF}$, can also be described as $\text{F}^- \cdots \text{H}^+ \cdots \text{F}^-$, thus its synonym, the proton-coupled bifluoride anion. With a $\text{F}^- \cdots \text{HF}$ bond strength of 45.8 ± 1.6 kcal/mol (1.99 eV) in the gas phase,²⁰ it is the strongest known hydrogen bond. Even a modest fraction of its gas-phase strength would be easily enough to supply the needed transition state stabilizations discussed above. Interestingly, the $\text{F}^- \cdots \text{HF}$ hydrogen bond strength in aqueous solution is only ~ 0.8 kcal/mol.²¹

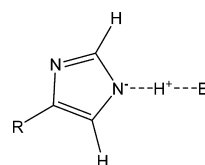
Proton-coupled bicarboxylates top the list as the earliest and still the best-studied systems suspected of forming

LBHBs in the vicinity of the active sites of enzymes.^{22–24} These hydrogen-bonded couples can be depicted as



and they can be abbreviated by the general formula $\text{X}^- \cdots \text{HX}$. Proton-coupled bicarboxylates appear in 16% of all protein X-ray structures.²⁵ There are at least five X-ray structures showing short (and therefore strong) hydrogen bonds between an enzyme carboxylate and a reaction intermediate or transition state analogue bound at the enzyme active site; four of these hydrogen bonds are 2.2 or 2.3 Å long, whereas one is 2.5 Å long.^{26,27} We consider these structures to be the best *de facto* evidence of the existence of low-barrier hydrogen bonds stabilizing high-energy reaction intermediates at enzyme active sites. Proton-coupled bicarboxylates are believed to be important components of the active sites of the aspartic acid proteases, e.g., HIV-1 protease.^{27–29} Carboxylates figure prominently in the LBHB enzymatic story in part because all negative charges on proteins are carboxylates.

Another system that is implicated in the formation of LBHBs in the vicinity of the active sites of enzymes is imidazole. In the early days of the LBHB story, the seminal work of Knowles had shown that the neutral imidazole side chain of His-95, acting as the general acid, stabilized the enediolate intermediate (E^-) in the reaction catalyzed by triosephosphate isomerase.^{4,5} This hydrogen-bonded couple can be depicted as



What made imidazole's role as an acid so astonishing was the fact that the pK_a of imidazole (in water) is 14. This apparent dilemma provided among the first clues that LBHBs might be playing important roles in enzyme catalysis.

Given the importance of carboxylates and imidazole in the LBHB story, it is important to know the strengths of their hydrogen-bonded couples in the gas phase (*in vacuo*) to establish their maximal possible values. Unfortunately, the debate over the possible role of strong, low-barrier hydrogen bonds in stabilizing reaction intermediates at enzyme active sites has taken place in the absence of an awareness of the upper limits to the strengths of low-barrier hydrogen bonds involving amino acid side chains. To help fill this gap, we have utilized anion photoelectron spectroscopy to measure the hydrogen bond strengths of the formate–formic acid, acetate–acetic acid, and imidazolide–imidazole, anion–neutral, intermolecular, hydrogen-bonded complexes (couples) *in vacuo*. To make a uniform comparison, we also measured the hydrogen bond strength of HF_2^- , i.e., the $\text{F}^- \cdots \text{HF}$ anion–neutral interaction energy, using this same experimental technique. Proton-coupled identical pairs have been used because bases with the same pK_a values form the strongest low-barrier hydrogen bond, and the purpose of this work is to establish an upper limit for the strength of low-barrier hydrogen bonds involving amino acid side chains.

■ EXPERIMENTAL PROCEDURES

Anion photoelectron spectroscopy is conducted by crossing a mass-selected beam of negative ions with a fixed-frequency

photon beam and energy-analyzing the resultant photodetached electrons. Photodetachment transitions occur between the ground state of a mass-selected negative ion and the ground and energetically accessible excited states of its neutral counterpart. This process is governed by the energy-conserving relationship $h\nu = \text{EBE} + \text{EKE}$, where $h\nu$ is the photon energy, EBE is the electron binding energy, and EKE is the electron kinetic energy. Measuring electron kinetic energies and knowing the photon energy provide electron binding (photodetachment transition) energies. Because these are vertical transitions, their relative intensities are determined by the extent of Franck–Condon overlap between the anion and its corresponding neutral. Our apparatus consists of a laser vaporization anion source, a linear time-of-flight mass spectrometer for mass analysis and mass selection, a momentum decelerator, a magnetic bottle electron energy analyzer, and an ArF excimer laser. The magnetic bottle has a resolution of ~ 50 meV at an EKE of 1 eV. In these experiments, photoelectron spectra were recorded with 193 nm (6.42 eV) photons. The photoelectron spectra were calibrated against the well-known transitions of atomic Cu^- . A detailed description of the apparatus has been reported elsewhere.³⁰

To produce the fluoride, hydrogen bifluoride; formate, formate–formic acid; and acetate, acetate–acetic acid anions, a small amount of sample (*S*-pentafluorobenzene, formic acid, or acetic acid, respectively) was entrained in helium (~ 60 psi) and expanded through the nozzle orifice (0.79 mm diameter) of a pulsed (10 Hz) valve (General Valve Series 9) in a high-vacuum chamber (10^{-6} Torr). To produce the imidazolide and imidazolide–imidazole anions, imidazole was placed in a small oven (~ 30 °C) attached to the front of the pulse valve, where helium (~ 60 psi) was expanded over the sample in a vacuum chamber. Just outside the orifice of the pulse valve, or in the case of imidazole just outside the orifice of the oven, low-energy electrons were produced by laser/photoemission from a pulsed Nd:YAG laser beam (10 Hz, 532 nm) striking a translating, rotating, copper rod (6.35 mm diameter). Negatively charged anions were then pulse-extracted into the spectrometer prior to mass selection and photodetachment.

RESULTS

In the systems studied here, the $\text{X}^- \cdots \text{HX}$ anionic complexes are bound, while the corresponding $\text{X} \cdots \text{HX}$ neutral complexes produced as a result of photodetachment are likely to be unbound. Figure 1 illustrates this situation schematically, where $\text{EA}(\text{X})$ is the adiabatic electron affinity of X, E_{asym} is the energy from the ground state of the $\text{X}^- \cdots \text{HX}$ anionic complex, i.e., HX_2^- , to the $\text{X} + \text{HX} + e^-$ energy asymptote, and $D(\text{X}^- \cdots \text{HX})$ is the dissociation energy of $\text{X}^- \cdots \text{HX}$ separating into $\text{X}^- + \text{HX}$, i.e., the hydrogen bond strength of the X^-/HX couple. Thus, $D(\text{X}^- \cdots \text{HX}) = E_{\text{asym}} - \text{EA}(\text{X})$.

In anion photoelectron studies of six hydrogen bihalide anions, HX_2^- , where here X denotes both homogeneous and heterogeneous combinations of the halogen atoms, Cl, Br, and I, Neumark found the $\text{X} \cdots \text{HX}$ neutral complexes, resulting from photodetachment of HX_2^- , to be unbound.^{31,32} Importantly, inspection of his photoelectron spectra shows that E_{asym} values, which in these particular cases are known from tabulated $\text{EA}(\text{X})$ and $D(\text{X}^- \cdots \text{HX})$ values, usually lie only ~ 0.2 eV above the EBE value of the photoelectron intensity onset, E_{OS} , in the corresponding HX_2^- photoelectron spectra. (The low-intensity “tail” between E_{asym} and E_{OS} was likely due to photodetachment of vibrationally excited HX_2^- anions, i.e., hot bands.)

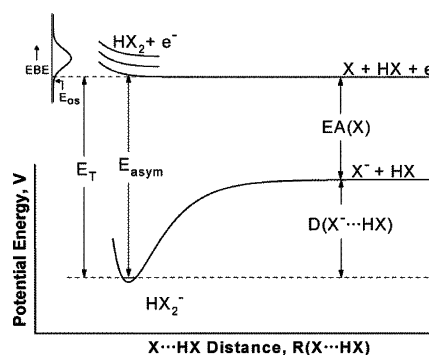


Figure 1. Schematic representation of the energetics of anion photoelectron (photodetachment) spectroscopy as applied to HX_2^- . Symbols are defined in the text.

Furthermore, if one defines a photoelectron intensity threshold, E_T , by extrapolating a straight line to the baseline from high on the low-EBE side of the lowest-EBE band in each spectrum, then the difference between E_{asym} and E_T becomes even smaller than that between E_{asym} and E_{OS} . Thus, the photoelectron spectra of hydrogen bihalide anions, which are in many ways analogous to the systems being studied here, support the approximation that $E_{\text{asym}} \cong E_T$. With this, we obtain the working relationship $D(\text{X}^- \cdots \text{HX}) \cong E_T(\text{HX}_2^-) - \text{EA}(\text{X})$.

In this work, we measured the photoelectron spectra of X^- and $\text{X}^- \cdots \text{HX}$ for each of the LBHB candidate systems of interest. Upon determination of $\text{EA}(\text{X})$ and $E_T(\text{HX}_2^-)$ values, their differences provided values of $D(\text{X}^- \cdots \text{HX})$, these being the sought-after hydrogen bond strengths of specific X^-/HX couples. In determining $\text{EA}(\text{X})$ values from photoelectron spectra of X^- anions, we benefited from previous photodetachment and photoelectron studies of the fluorine atomic anion,³³ the formate anion,³⁴ the acetate anion,³⁵ and the imidazolide anion.³⁶ These studies assigned the origin transitions in their respective X^- photoelectron spectra, thereby providing accurate $\text{EA}(\text{X})$ values. While our photoelectron spectra of these same X^- anions were recorded at a lower resolution, they are fully consistent with those previously recorded, allowing us to locate the EBE value of their origin transitions on the spectral profiles observed in this study. Values of $E_T(\text{HX}_2^-)$ were determined as described above by extrapolation along the low-EBE side of the lowest-EBE spectral band in our HX_2^- photoelectron spectra.

Figure 2 presents the photoelectron spectra of corresponding sets of X^- and HX_2^- species measured in this work. In each panel, the spectrum of X^- is positioned above that of HX_2^- , but on the same energy scale, facilitating a pictorial depiction of $D(\text{X}^- \cdots \text{HX})$ as the energy difference between specific points (see vertical tick marks) on the two photoelectron spectra, these points designating the values of $\text{EA}(\text{X})$ and $E_T(\text{HX}_2^-)$, respectively. For this reason, the length of the horizontal arrow in each panel is a measure of the hydrogen bond strength, $D(\text{X}^- \cdots \text{HX})$, of its corresponding HX_2^- species, i.e., of the X^-/HX couple. Referencing the hydrogen bond strengths of the three enzymatic model systems studied here to that of HF_2^- , by using the same experimental method for all four, puts all these measurements on a common footing and provides confidence in comparing the results. Thus, panels a–d of Figure 2 successively present the photoelectron spectra of the fluorine anion, F^- , and the fluoride–hydrogen fluoride, hydrogen-bonded complex, $\text{F}^-(\text{HF})$, i.e., HF_2^- ; the photoelectron spectra of the formate anion, Fo^- , and the formate–formic acid, hydrogen-bonded

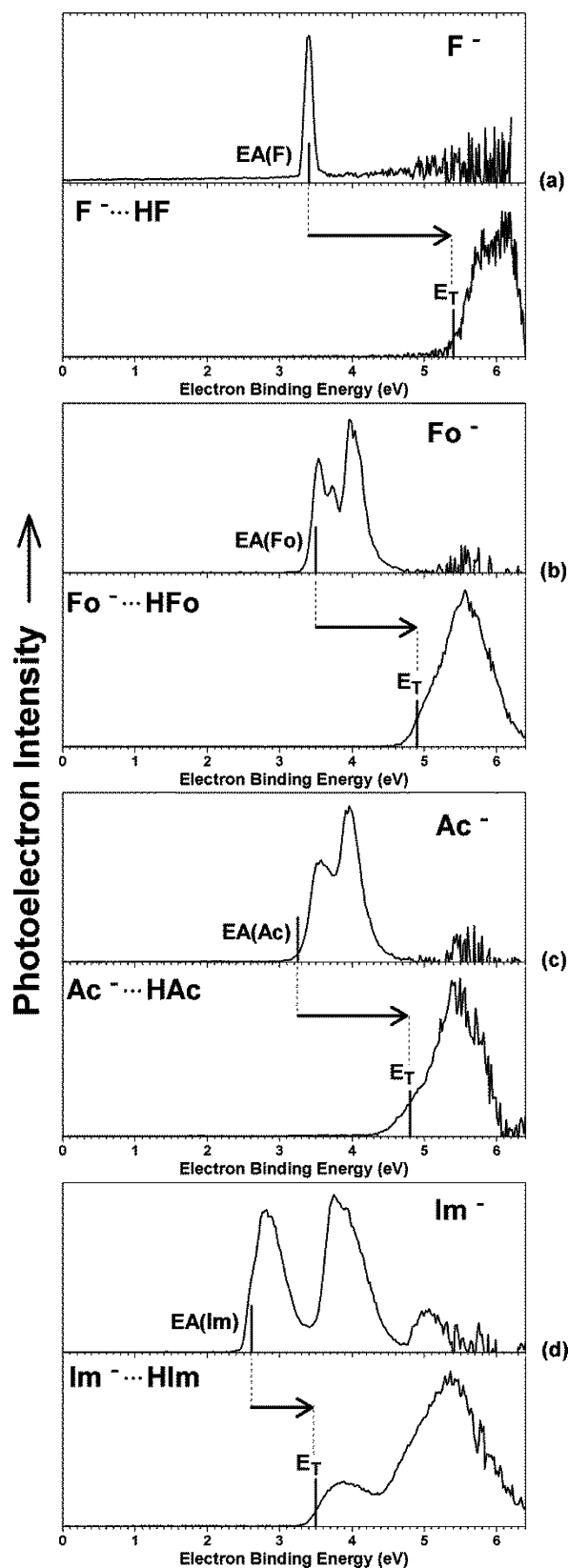


Figure 2. Anion photoelectron spectra of the four corresponding sets of X^- and HX_2^- species measured in this work. All spectra were calibrated against the well-known photoelectron spectrum of Cu^- , the anion of the copper atom.

complex, $Fo^-(HFo)$; the photoelectron spectra of the acetate anion, Ac^- , and the acetate–acetic acid, hydrogen-bonded

complex, $Ac^-(HAc)$; and the photoelectron spectra of the imidazolid anion, Im^- , and the imidazolid–imidazole, hydrogen-bonded complex, $Im^-(HIm)$. Table 1 presents values of $EA(X)$,

Table 1. Values Leading to Hydrogen Bond Strengths of X^-/HX Couples, i.e., $D(X^- \cdots HX)^a$

$X^- \cdots HX$ system	$EA(X)$ from literature	$E_T(HX_2^-)$ from this work	$D(X^- \cdots HX)$		approximate % of $F^- \cdots HF$ HB strength
			this work	previous work	
$F^- \cdots HF$	3.40 ^b	5.4	2.0	2.0 ^f	100
$Fo^- \cdots HFo$	3.50 ^c	4.9	1.4	1.6 ^g	70–80
$Ac^- \cdots HAc$	3.25 ^d	4.8	1.6	1.3 ^g	65–80
$Im^- \cdots HIm$	2.61 ^e	3.5	0.9	1.1 ^h	45–60

^aEnergies are presented in units of electronvolts. Uncertainties are ± 0.1 eV or less. ^bFrom ref 33. ^cFrom ref 34. ^dFrom ref 35. ^eFrom ref 36. ^fFrom ref 20. ^gFrom ref 37. ^hFrom ref 38.

$E_T(HX_2^-)$, and $D(X^- \cdots HX)$ for each of the four systems that we studied here, where $D(X^- \cdots HX)$ is the measured hydrogen bond strength for that particular X^-/HX couple.

DISCUSSION

In the past, the dissociation energies of the HX_2^- systems studied here have also been determined in the gas phase by Wenthold and Squires, using energy-resolved, collision-induced dissociation (CID),²⁰ and by Meot-Ner (Mautner), using high-pressure mass spectrometry and van't Hoff plots.^{37,38} The CID measurement of the hydrogen bond strength of HF_2^- gave a value of 1.99 eV (45.8 kcal/mol), whereas the HF_2^- hydrogen bond strength measured in our work was 2.0 eV (46 kcal/mol). The thermodynamic–van't Hoff plot determinations of the hydrogen bond strengths of $Fo^-(HFo)$, $Ac^-(HAc)$, and $Im^-(HIm)$ were reported as 1.60 eV (36.8 kcal/mol), 1.27 eV (29.3 kcal/mol), and 1.14 eV (26.4 kcal/mol), respectively, whereas the hydrogen bond strengths of the corresponding species measured in our spectroscopic work were 1.4 eV (32 kcal/mol), 1.6 eV (37 kcal/mol), and 0.9 eV (21 kcal/mol), respectively. What is important about these complementary measurements is that their values, while measured using different techniques, are comparable. They support one another by yielding the same approximate values for corresponding hydrogen bond strengths. It is interesting to note that the participants in the LBHB debate were apparently unaware of the van't Hoff plot values.

The core result of both the work presented here and previous work is that all three of the enzymatically relevant model systems considered here exhibit upper limit (gas phase), hydrogen bond strengths that are very strong. According to our measurements, the hydrogen bond strengths of the formate–formic acid, acetate–acetic acid, and imidazolid–imidazole complexes are 70, 80, and 45%, respectively, of the strength of the fluoride–hydrogen fluoride complex, HF_2^- , with values from previous measurements giving comparable percentages. Thus, the carboxylate and imidazolid, intermolecular hydrogen-bonded X^-/HX couples queried here in the gas phase do indeed have hydrogen bond strengths that are comparable to that of the F^-/HF hydrogen-bonded couple. Furthermore, it is also interesting to note that in a gas-phase photoelectron study by Wang,³⁹ the shift between *trans*- and *cis*- $HO_2CCH=CHCO_2^-$ (the fumaric/maleic acid monoanion) spectra revealed the *intramolecular* hydrogen bond strength in the *cis*

isomer (hydrogen maleate) to be 21.5 kcal/mol, which is 47% of the hydrogen bond strength of HF_2^- .

The threshold hydrogen bond strengths needed to account for the stabilization of enzymatic intermediates have been variously estimated to lie between 7 kcal/mol^{6,7} (in the case of imidazole) and 20 kcal/mol.^{8,19} The gas-phase, hydrogen bond strengths that we measured for the Fo^- (HFO), Ac^- (HAc), and Im^- (HIm) complexes are 32, 37, and 21 kcal/mol, respectively. The lower threshold value of 7 kcal/mol is 22, 19, and 33% of the measured hydrogen bond strengths of these complexes, respectively, whereas the higher threshold value of 20 kcal/mol is 63, 54, and 105% of these same strengths, respectively. If there were to exist enzyme active site environments that allowed hydrogen-bonded couples to retain significant percentages of their gas-phase (isolated) strengths, and if these exceeded the pertinent threshold values, then ionic hydrogen bonding might well figure prominently in facilitating enzymatic rate enhancements.

By definition, an environment in which a hydrogen-bonded couple has no opportunity to form alternative hydrogen bonds would preserve its strength, viz., *in vacuo* (gas phase). In solution (liquids), however, where there may be many competing hydrogen bonding interactions, the strength per hydrogen-bonded couple would be lowered. One can also envision a quasi-solid state regime of limited molecular mobility, lying between these extremes. There, both the opportunities for forming alternative hydrogen bonds (the degree of competition for forming them) and the corresponding strengths of their hydrogen bonds would be intermediate between those of gases and liquids. The envisioned relationship between hydrogen bond strength and the extent of competition with regard to these three environments is illustrated schematically in Figure 3.

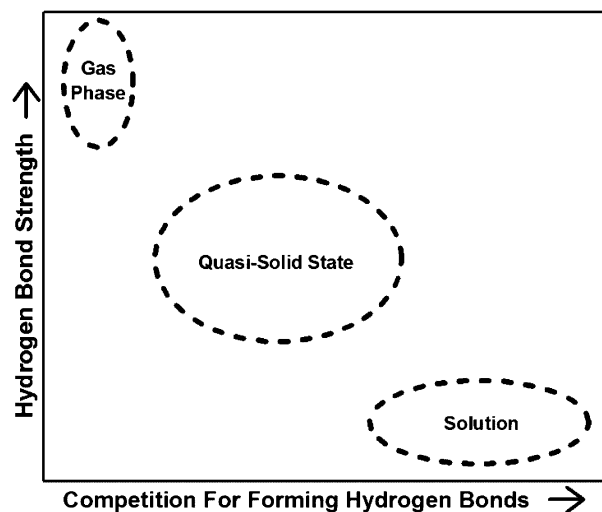


Figure 3. Schematic representation of the relationship between hydrogen bond strengths and the degree of competition for forming hydrogen bonds in three different environments.

Thus, it is plausible that some enzyme active site environments may correspond to this intermediate case, giving them the possibility of maintaining exceptionally strong hydrogen bonds. Even so, such strong hydrogen bonds would be only part of the story, because other factors, such as local geometry and protein strain, are also expected to play important roles. This work does not prove the validity of the LBHB hypothesis. However, through gas-phase (*in vacuo*) measurements, it does

provide upper limits to the possible strengths of several enzymatically relevant hydrogen bond couples. It also suggests a framework for describing how some enzyme active sites might preserve a substantial portion of that strength for their use in catalysis.

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Notes

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ABBREVIATIONS

TIM, triosephosphate isomerase; LBHB, low-barrier hydrogen bond; $h\nu$, photon energy; EBE, electron binding energy; EKE, electron kinetic energy; EA(X), adiabatic electron affinity of X; E_{asym} , energy from the ground state of $\text{X}^- \cdots \text{HX}$ to the $\text{X} + \text{HX} + e^-$ energy asymptote; $D(\text{X}^- \cdots \text{HX})$, dissociation energy of HX_2^- ; E_{OS} , photoelectron intensity onset; E_{T} , photoelectron intensity threshold; Fo^- , formate anion; Ac^- , acetate anion; Im^- , imidazolate anion; CID, collision-induced dissociation.

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