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Low barrier hydrogen bonds in protein structure and function

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Abstract

Low-barrier hydrogen bonds (LBHBs) are a special type of short hydrogen bond (HB) that is characterized by the equal sharing of a hydrogen atom. The existence and catalytic role of LBHBs in proteins has been intensely contested. Advancements in X-ray and neutron diffraction methods has revealed delocalized hydrogen atoms involved in potential LBHBs in a number of proteins, while also demonstrating that short HBs are not necessarily LBHBs. More importantly, a series of experiments on ketosteroid isomerase (KSI) have suggested that LBHBs are significantly stronger than standard HBs in the protein microenvironment in terms of enthalpy, but not free energy. The discrepancy between the enthalpy and free energy of LBHBs offers clues to the challenges, and potential solutions, of the LBHB debate, where the unique strength of LBHBs plays a special role in the kinetic processes of enzyme function and structure, together with other molecular forces in a pre-organized environment.

Keywords

LBHB; Enzyme catalysis; Short HB; Sub-angstrom resolution X-ray crystallography; Neutron diffraction; NMR downfield shift

1. Introduction: Theory and historical perspective

Hydrogen bonds (HBs) play a fundamental role in biological chemistry. Whereas different types of HBs and their properties have been extensively studied in small molecules, the fast development of macromolecular X-ray crystallography in the early 1990s, combined with the use of transition state analogs (TSAs) and NMR, led to the discovery of short HBs in a number of enzymatic systems including, most notably, zinc metallopeptidases [1–3], triose-phosphate isomerase (TIM) [4,5], and serine proteases such as trypsin, chymotrypsin, and subtilisin (Table 1) [6]. Compared with standard HBs with a heteroatom distance of 2.8–3.0 Å, these short HBs generally have a length of approximately 2.5 Å. Based on the studies of small molecules, such short HBs are often formed between functional groups with comparable pKa's, and have a zero point energy of the shared hydrogen higher than the barrier height energy for proton transfer. Thus, these special short HBs were given the name

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low-barrier hydrogen bonds (LBHBs) by Cleland (Fig. 1) [7]. The unique feature of LBHBs results in a delocalized proton equally shared by the two polar heteroatoms in a symmetrical fashion. This is similar to a single-well HB that is even shorter (~2.3 Å) but occurs mostly in some special cases of small molecules. Partially delocalized protons are also observed in asymmetric, short HBs as intermediate types between a standard HB and a symmetrical LBHB. Due to enhanced proton sharing, these asymmetric HBs, like LBHBs, can be stronger than a normal HB. For the discussion herein, such short HBs with partially delocalized hydrogens will also be regarded as LBHBs.

The interest surrounding LBHBs for enzyme studies is due to its extraordinary strength, which can be ~5–10 times stronger than a standard HB in the gas phase [57]. The initial hypothesis described a transient species at the reaction transition state (TS) when the pKa's of the donor and acceptor of a HB, involved in general base or acid catalysis, are equal to each other, thus providing an exceptionally strong interaction and serving as the driving force to lower the TS energy [57]. Despite the enormous appeal due to its ability to provide a simple answer to the mystery of enzyme catalysis, the concept of LBHBs has been intensely debated from the onset for several reasons. First, the existence of LBHBs in macromolecules was doubted due to the inability to directly observe hydrogen atoms in protein crystal structures in the early days of X-ray crystallography, and there were questions about the presence of these short HBs being potential artifacts of crystal packing [58]. Second, the strength of the LBHB was disputed as some studies predicted that LBHBs are much weaker in water and protein microenvironments compared to gas phase calculations, and more on the order of a typical HB [59,60].

Over the last two decades, the advancement of X-ray and neutron crystallography has elucidated unprecedented details of macromolecular HBs, while new tools of chemical biology have enabled in-depth investigation of individual HBs in protein structure and function. These studies provide important answers about the existence and strengths of LBHBs in the protein microenvironment and reveal potential new roles of LBHBs in protein structures.

2. Detection

Due to the inherently transient nature of potential LBHBs in enzyme catalysis, and the need to pinpoint the hydrogen atom position, detecting these unique HBs has understandably proven to be a challenge, particularly in the context of protein structures. Small molecule ligands, including TSAs and non-covalent inhibitors, have been used to reveal potential LBHBs, while some short HBs have been captured in apo proteins. While NMR and X-ray/neutron crystallography represent the main techniques to analyze LBHBs, infrared and kinetic isotope effects are also useful experimental techniques [61]. All of these methods continue to be utilized in the study of LBHBs, but the most progress has been made in the area of crystallography due to the dramatic increase in the quantity and resolution of protein structures. In addition, computational modeling has become accurate enough to offer unique insights beyond the limitations of experimental techniques.

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2.1. Prevalence of short HBs in protein structures

In the last three decades since the LBHB hypothesis was initially formulated, the Protein Data Bank (PDB) has seen its depositions increase from ~500 in 1990, to more than 160,000 to date. Investigation of 1,663 high quality protein crystal structures (<1.1 Å resolution) revealed 3,314 HBs with lengths <2.6 Å and 15,968 HBs with lengths <2.7 Å [62]. These HBs include interactions among both side chain and backbone functional groups, echoing an earlier statistical analysis [63]. Other studies focused on short HBs between pairs of side chain carboxylate groups from aspartate or glutamate residues, found in approximately 14% of protein structures and with an average length of 2.542 Å [50]. Short HBs are also frequently observed in protein-ligand complexes, such as a 2.35 Å HB between Glu52 and the substrate hydroxyl group in a recently determined X-ray complex crystal structure of aminoglycoside nucleotidyl transferase 4′(ANT4') from *Staphylococcus aureus* [55]. Some of these macromolecular HBs, especially between carboxylate pairs, can potentially be LBHBs, while other short HBs, including those involving only protein backbone atoms, do not appear to satisfy the conditions of matching pKa's for LBHB formation. Some of the short HBs may partly result from local geometric restraints [63], including crystal packing artifacts, particularly under commonly used cryogenic conditions that can cause further shrinkage of crystal volume and protein structures [64,65]. In addition, positional errors in crystal structures, ~0.2 Å for well-ordered atoms at ~2 Å diffraction resolutions [1,42], can further complicate the use of the HB length in inferring its property. For this reason, some early examples of proposed LBHBs, such as the one in ovotransferrin, may warrant additional scrutiny (Table 1). Taken together, these observations suggest a range of short HBs are available in protein crystal structures, and the majority of them are most likely not LBHBs.

2.2. Hydrogen/deuterium atoms revealed by crystallography

The developments in high-intensity synchrotron X-ray sources and detector technology have dramatically improved the resolution limit of protein crystals. Sub-Angstrom resolution X-ray diffraction reduces the positional error to <0.1 Å [61], and also enables the determination of hydrogen atom positions. Additionally, new neutron crystallography facilities, such as the spallation neutron source (SNS) and the macromolecular neutron diffractometer (MaNDi) at Oak Ridge National Laboratory, have made neutron diffraction analysis more accessible to proteins [66]. An increasing number of sub-Angstrom resolution X-ray crystal structures and high-resolution neutron structures have revealed unprecedented details of short HBs, particularly the hydrogen atom positions. These structures demonstrate that LBHBs with delocalized hydrogens exist in proteins, but also show that short HBs can have localized hydrogens. In addition, while most of these LBHBs have been validated by other methods such as NMR and QM/MM calculations, at least one LBHB seems to have been caused by crystal artifacts [38,39].

The first instance of a delocalized hydrogen in a macromolecular structure was observed between Asp32 and His64 in the 0.78 Å resolution X-ray crystal structure of the serine protease subtilisin, crystallized at pH 5.9 [27]. The Asp-His HB in the catalytic triad of serine proteases has been proposed to enhance the ability of the histidine residue to deprotonate the catalytic serine during the attack on the peptide substrate. The low

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crystallization pH for the structure resulted in a positively charged histidine, mimicking the TS after the deprotonation of the catalytic serine. The hydrogen shared between Asp32 and His64 is 1.2 Å from His64Nδ1 and 1.5 Å from Asp32Oδ2, with the heteroatom distance of 2.62 Å. In contrast, in another ultrahigh resolution serine protease X-ray crystal structure, the 0.82 Å resolution α-lytic protease (αLP) complexed with a boronic acid TSA, an equally short HB (2.64 Å) between His57Nε2 and the boronic acid oxygen has the shared hydrogen localized on the histidine, while the Asp-His HB has a standard length of 2.73 Å [9]. These two examples underscore the caveat of using HB length alone to infer the identity of the HB. Similarly, a 1.15 Å resolution crystal structure of the human protein DJ-1 illustrates a short HB (2.49 Å) between Glu15 and Asp24, but with the hydrogen localized on Asp24 [50]. Together with the survey of the PDB, the ultrahigh resolution crystal structures demonstrate that a short HB length does not necessarily equate to a LBHB. This is further demonstrated by theoretical calculations on the short HB between Asp148 and the chromophore hydroxyl group in the S65T/H148D mutant of green fluorescent protein (GFP) [41]. Although this HB has a short length of 2.45 Å in a series of high resolution crystal structures, the computational analysis suggested that the shared hydrogen is localized [42].

Recent X-ray crystal structures have also revealed two examples of hydrogens equally shared by the two heteroatoms. The first example is CTX-M-14 Class A β-lactamase bound by a tetrazole-based non-covalent inhibitor [48]. Class A β-lactamases are bacterial enzymes that hydrolyze penicillins and other β-lactam antibiotics, using a catalytic serine, and are a major cause of bacterial antibiotic resistance. The HB between the catalytic Ser70 and the purported base, Lys73, is 2.53 Å in length, and the hydrogen atom is 1.3 Å from both Ser70Oγ and Lys73Nζ (Fig. 2A). In another case, the 0.97 Å resolution complex X-ray crystal structure of human transketolase with a covalent intermediate, a short HB with a length of 2.56 Å is observed between Glu366 from one monomer and Glu160 from the other monomer of the biological dimer, with the hydrogen again ~1.3 Å from both residues [46].

Whereas a hydrogen atom only scatters X-rays weakly due to its lone electron and yields a negative signal in neutron diffraction, deuterium has coherent neutron scattering cross sections comparable to carbon, nitrogen, and oxygen [67]. Thus, given suitable resolutions, neutron diffraction provides a technique that is better equipped to pinpoint the location of the shared deuterium atom in a potential LBHB. In the 1.5 Å resolution neutron crystal structure of photoactive yellow protein (PYP) [37], a partially delocalized deuterium nucleus is visualized in the short HB (2.58 Å) between the carboxylate of Glu46 and the phenolic oxygen of the chromophore p-coumaric acid (pCA), with the deuterium 1.2 and 1.4 Å away from the two hydrogen bonding functional groups. In the 2.20 Å resolution neutron structure of aminoglycoside N3-acetyltransferase–VIa (AAC-VIa) with sisomicin [54], Glu192Oε2 and His189Nδ1 form a short HB of 2.57 Å, with the deuterium nuclei also 1.2 and 1.4 Å away from Glu192Oε2 and His189Nδ1 respectively (Fig. 2B). Recently, an equally shared deuterium atom was also observed in a number of neutron crystal structures including methylthioadenosine nucleosidase [49], aspartate aminotransferase [51], and concanavalin A [52]. Interestingly, the deuterium atom in these HBs is not located colinearly with the hydrogen bonding heteroatoms due to their relative positions. As a result, although the deuterium is equidistant to the two heteroatoms, the distances to the heteroatoms are longer (~1.7–1.8 Å) in some of these structures than other potential LBHBs (1.3–1.4 Å). Without

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additional support from NMR or QM/MM calculations, these new examples represent unique LBHB geometry that will need to be further investigated.

Although the high resolution X-ray and neutron structures have demonstrated potential delocalized H/D atoms, accurately determining the H/D positions remains a challenge. For the majority of the sub-Angstrom resolution X-ray structures, the shared hydrogen of these short HBs is only visible in the Fo-Fc map, and its position was not refined. The 0.83 Å resolution αLP X-ray crystal structure underwent rigorous refinement, but even with some of the lowest R and R_{free} values among all crystal structures, unstrained refinement of hydrogen atoms led to significant deviations of O–H and N–H bond lengths by as much as ~0.3 Å for some standard HBs [9]. Several potential LBHBs from various proteins have also been subjected to computational analysis. In the cases of bacterial CTX-M-14 β-lactamase and human transketolase, QM/MM calculations confirmed that the proton transfer barrier is low [46,48]. For PYP, although similar results were obtained [39], it was discovered that crystal packing likely resulted in a neutral state of Arg52, which lies between the chromophore pCA and the bulk solvent [38]. In solution studies by NMR and computer simulations, where increased protein dynamic behavior would lead to a positively charged Arg52, this LBHB was not observed [38]. These results caution against inferring biological functions from LBHBs observed only by crystallography. Another potential crystal artifact can originate from the averaged nature of these structures, which represent the mean coordinates and features of an enormous number of conformationally similar molecules inside the crystal. For a LBHB, the delocalized electron/nuclear density is caused by the movement of the proton and the distortion of electron density inside one single HB configuration. However, for short HBs with the shared hydrogen atom alternately localized on the two functional groups, the averaging effects of crystal structures may result in misleading delocalized densities that also appear in the middle of the HB, even when the proton transfer barrier is high. Such caveats further underscore the need to use additional methods to confirm potential LBHBs observed by crystallography.

2.3. NMR

Much of the work describing the existence of LBHBs has relied on NMR, with the first early examples in the analysis of chymotrypsin [6,68] and TIM [4]. LBHBs exhibit a downfield chemical shift for proton spectra of 16–20 ppm, corresponding to a deshielded proton in short HBs [69]. Unlike crystallography, NMR allows the detection of potential LBHBs in a more biologically relevant environment. New examples of potential LBHBs identified by NMR include a HB between Asp32 and the inhibitor hydroxyl group in the complex of endothiapepsin [33], and most recently between His51-Asp75 in the catalytic triad of NS3 serine protease from Dengue virus type II [53]. However, theoretical analysis has suggested that the unique downfield shift can also originate from short ionic HBs where the shared hydrogen is localized [70]. This was supported by later calculations on the Asp-His short HB in chymotrypsin [17] and trypsin [40]. Similar to the calculations on GFP [42], these results indicate that the short HB length cannot be used to validate a LBHB, no matter whether it is determined by crystallography or NMR.

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3. Strengths

Exactly quantifying the strength of a LBHB is at the crux of the debate surrounding its biological role. Relative to an average of 1–3 kcal/mol for the strength of a standard HB in protein structures [71], the range of potential LBHB strength for enzyme catalysis has been estimated to be approximately 20–30 kcal/mol on the high end [57], and 5–7 kcal/mol on the more conservative side [72]. The complexity of quantifying a single HB in a macromolecule is daunting due to the difficulty of isolating the effect of a specific HB from other interactions during experimental perturbations (e.g., mutagenesis), and the influence of the dielectric constant of the environment. Adding to the confusion is the ambiguity in the definition of 'strength' [73], and the use of a macro parameter of dielectric constant itself in a protein microenvironment where every individual atom is accounted for. Strictly speaking, 'strength' refers to the enthalpy of the HB formation involving only the two hydrogen bonding partners in vacuum. But what is commonly measured and discussed is the free energy of HB formation, which often involves the environment (such as the solvent).

The LBHB formed between acetate and acetic acid (i.e., side chains of aspartate and glutamate residues) can be as strong as 29.3 kcal/mol in the gas phase [74], in comparison to ~3–5 kcal/mol for a standard HB in the same environment [71,75]. However, studies using small molecules have demonstrated that, even though the HB length correlates with the pKa of the two hydrogen bonding groups irrespective of the solvent, the free energy of HB formation strongly depends on the environment [76]. In water, the free energy does not change significantly as the pKa decreases and the HB length shortens. But in aprotic solvent such as chloroform, the free energy increases significantly at approximately 1 kcal/mol per 0.03 Å change and extrapolates to approximately −12 kcal/mol when pKa = 0, demonstrating the significant influence of the environment. However, the non-polar solvent with its homogeneous dielectric constant cannot truly represent the heterogeneous microenvironment of a protein active site. It is therefore important to analyze short HBs directly inside the protein.

Despite the experimental challenges, several methods have been applied to estimate the 'strength' of specific short HBs in proteins, especially in the model system of ketosteroid isomerase (KSI) (Fig. 3). Although the results sometimes provide only relative changes or upper/lower limits, these incomplete pictures nevertheless offer valuable insights into some unique properties of macromolecular short HBs. Based on the hydrogen exchange behavior in NMR analysis, an early study of KSI estimated the strength of the short HB between Tyr14 and the substrate analog to be >7.1 kcal/mol [23]. Similar experiments later determined the activation enthalpy for several LBHBs in KSI and chymotrypsin to be in the range of 12–20 kcal/mol [16,77]. One of the most in-depth investigations of macromolecular short HB strengths, performed by the Herschlag group, used KSI and a series of phenolates with varying pKa's, modulating the short HB between the tyrosine and the phenolate oxygen [78]. The changes in binding affinity showed a modest influence of pKa on the binding free energy (G = –0.2 kcal/mol/pKa unit), but a significant effect on enthalpy (H = –2.0 kcal/mol/pKa unit). As the enthalpic difference most likely originated from the changes in the HB itself, these results suggest that short HBs, particularly LBHBs, can be

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significantly stronger than standard HBs, but do not offer additional stability in terms of free energy when the environment is taken into account.

4. Biological functions

4.1. Contribution to enzyme catalysis

The interest in LBHBs was originally focused on their potential role in enzymatic reactions, and heated debates took place in which this role ranged from a driving force to being anti-catalytic [57,79]. There have been several attempts to quantify the contribution of specific LBHB candidates to reaction rates. Disrupting the His-Asp short HB in subtilisin BPN' through Asp- >Cys mutation resulted in only a 50 fold decrease in k_{cat}, leading to the suggestion that this HB contributes at most 2.2 kcal/mol to the reduction in TS energy [80]. However, studies on other proteins have found more than 103 fold decreases in k_{cat} when residues involved in short HBs were mutated [81,82]. As any mutation would normally impact multiple interactions, these results do not necessarily reflect the importance of the short HB in enzymatic reactions. However, an elegantly designed recent experiment used unnatural amino acid protein synthesis to incorporate tyrosine analogs in KSI, in order to systematically perturb the short HB [83]. The results showed that up to two unit changes in the tyrosine pKa had negligible effect on the reaction rate.

While studies such as KSI do not appear to support a special enzymatic role of specific potential LBHBs, these quantitative analyses at least do not exclude the possibility of such unique contributions by other LBHBs. The series of sub-Angstrom resolution crystal structures of CTX-M-14 Class A β-lactamase offer new clues to potential involvement of LBHBs in enzymatic reactions, especially related to general base catalysis where proton transfer takes place [48]. The studies of Class A β-lactamase benefit from a detailed mechanistic understanding of the reaction pathway afforded by QM/MM calculations [84]. The hydrolysis of β-lactam compounds proceeds through a covalent acyl-enzyme intermediate between the catalytic Ser70 and the carbonyl group of the β-lactam ring (Fig. 4). Based on computational predictions [84], two residues, Glu166 and Lys73, are involved in activating Ser70 in a concerted base mechanism. In this model, both Glu166 and Lys73 are charged in the apo enzyme, but substrate binding triggers a series of proton transfers and converts both residues to a neutral state. A neutral Lys73 then serves as the general base to deprotonate Ser70. The protonation states of Lys73 and Glu166 were confirmed by the 0.79 Å resolution apo structure of CTX-M-14 and the 0.89 Å resolution non-covalent complex structure with a tetrazole-based inhibitor [48]. Interestingly, as previously described, a LBHB was also present between Ser70 and Lys73 in the inhibitor complex, representing the first instance of a LBHB between a nucleophile and the general base where a proton is transferred from the former to the latter during the reaction. In addition, in a third 0.84 Å resolution acylation TSA structure with a boronic acid inhibitor, the potential Ser70-Lys73 LBHB is abolished, and the active site contains a positively charged Lys73 and a neutral Glu166, representing the state immediately after Lys73 deprotonates Ser70. Subsequently, the general acid, Ser130, transfers its proton to the substrate leaving group, the ring nitrogen, while taking a proton from Lys73. This results in a neutral Lys73 and a neutral Glu166 in the

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acyl-enzyme stage, as captured by another 0.83 Å resolution structure of CTX-M-14 with avibactam [85].

The four CTX-M-14 structures have important implications for the investigation of LBHBs, especially related to the use of small molecules. As evidenced by the protonation state change and LBHB formation in the non-covalent complex, ligand binding itself plays an important role in inducing the pKa change necessary for a LBHB between catalytic residues. The absence of the LBHB in the covalent boronic acid complex confirms the common knowledge that TSAs are not perfect mimics of the high energy reaction intermediate and demonstrates that the selection of the right compounds is crucial to capturing specific LBHBs. It is also interesting that QM/MM calculations predicted that general base catalysis takes place at the TS of the acylation reaction, whereas the general acid catalysis occurs during the collapse of the TS [84]. In the acylation TSA structure, a standard HB was observed between the purported general acid, Ser130, and the boronic acid oxygen mimicking the substrate leaving group, the ring nitrogen.

It may be possible to reconcile the quantitative results from KSI with a potential catalytic role of the Ser70-Lys73 LBHB. Various short HBs may form at different stages of the reaction, and not all of them take place at the TS. The static picture of a crystal structure, in the presence of a non-substrate ligand, does not directly tell us when exactly the short HB of interest occurs during the reaction, nor whether other short HBs can be captured with alternative ligands. For β-lactamase, it is conceivable that short HBs can also form between the general acid Ser130 and the substrate leaving group during the reaction, as well as between the TS oxyanion (Fig. 4, step III) and the backbone amide groups of Ser70 and Ser237, which constitute the oxyanion hole of β-lactamase, similar to the function of Tyr14 and Asp99 in tKSI (Fig. 3). These potential short HBs may contribute to the acylation reaction differently than the LBHB between Ser70 and Lys73 in the general base catalysis. For KSI, the general base, an aspartate residue (Asp38 in tKSI) has also been found to be crucial for the reaction (Fig. 3A) [86]. It is possible that the strength of the short HB involving the oxyanion hole Tyr14 might not be as important as hypothesized, even though the reverse can also be true. Furthermore, a network of HBs and other interactions contribute to the stabilization of the TS. Perturbations of some HBs may be compensated by other changes in this network. In the CTX-M-14 β-lactamase non-covalent complex, a short HB involving Glu166 and the catalytic water (2.52 Å) is also observed in the active site, although the shared hydrogen atom is localized [48]. Studies of the two HBs between the ligand oxygen atom and the protein side chains in the oxyanion hole of KSI and PYP have shown that perturbations shortening one HB can lead to the lengthening of the other [87]. Finally, the strength, or more precisely, the enthalpy of the LBHB, can be crucial to the kinetic process of enzymatic reactions in a pre-organized protein active site, where multiple molecular forces with the necessary strengths have to strike the right locations at specific reaction coordinates. After all, as approximated in the ultrahigh resolution X-ray crystal structures, what the shared hydrogen atom has shown is the distortion of electron densities on two functional groups involved in enzyme catalysis. Such details of enzymatic reactions are not captured in the free energy analysis in most of our current experimental approaches. The strength of LBHBs may also be especially important for a proton transfer process where a LBHB has to take place. It is conceptually appealing, and theoretically plausible, to

postulate that two functional groups would move closer to form a LBHB to facilitate the proton transfer, as their pKas change along the reaction coordinate. For such LBHBs at least, their unique strength does contribute to lowering the TS energy, because the TS barrier would be higher if the enthalpy of LBHB formation is the same as a standard HB.

4.2. Maintaining protein structural integrity

Aside from the potential catalytic role, the prevalence of short HBs, especially those involving carboxylate pairs, suggests that LBHBs may play a special structural role as well. In rhamnogalacturonan acetylesterase D192N mutant, a potential Asp75-Asp87 LBHB, with a length of 2.47 Å in the crystal structure, was also identified by NMR [43]. This Asp-Asp pair is located close to the active site and can potentially serve as a kinetic barrier to maintaining the structural integrity of the enzyme active site, which usually harbors many high energy features. Previous studies have demonstrated the importance of kinetic stability for αLP, which is thermodynamically unstable, yet is able to maintain its native state in solution [88]. However, it remains to be seen whether these short HBs between pairs of carboxylate side chains are indeed LBHBs, or rather short ionic HBs that can also be stronger than an average standard HB. Some of these HBs are partially exposed to solvent and are likely short ionic HBs, whereas others are buried deeper in the protein core and may contain delocalized hydrogens. For sub-Angstrom resolution CTX-M-14 crystal structures, an equally-shared hydrogen atom was observed in the short HB (2.47 Å in length) between Asp233 and Asp246, a pair of aspartate residues located near the active site and highly conserved in other Class A β-lactamases [48]. Like the LBHB in PYP, this short HB is shielded from the solvent by an arginine residue. Although it appears to be a LBHB in the crystal, it can be exposed to the solvent in solution and possibly exist as a short ionic HB. Nevertheless, the highly conserved nature and unique location of this aspartate pair suggests it can have a special structural role.

Another potential contribution of LBHBs to protein structures was recently revealed in the homodimeric complex of the aforementioned human transketolase, where a LBHB (2.56 Å in length) is observed between Glu366 from one monomer and Glu160 from the other monomer in each of the two active sites [46]. The two glutamate pairs in the two active sites are connected to one another through a series of HBs involving Glu165 and Gln367 from both monomers, and a number of water molecules. It is proposed that the LBHB, together with neighboring HB partners, may enable the Glu366-Glu160' pair to switch between a negatively charged and neutral state, and influence the corresponding glutamate pair in the neighboring active site through a potential 'proton wire' mediated by the other protein residues and water molecules. The E160Q mutation converts the LBHB to a standard HB and was shown to have a relatively modest 5× decrease in catalytic activity but abolished the positive cooperativity between the two active sites. Additionally, the T382E mutation outside the active site and acting through long-range effects, shortens the LBHB from 2.56 Å to 2.52 Å, while increasing both the activity and cooperativity.

4.3. Ligand binding

In addition to stabilizing reactive substrate functional groups in enzymatic reactions, LBHBs can also have non-catalytic roles during ligand binding. For bacterial periplasmic phosphate-

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binding proteins (PBPs) of the ABC-type transport system, a potential LBHB is formed between Asp62 and the phosphate molecule, and it is replaced by a potentially weaker short ionic HB in the arsenate complex, possibly explaining the selectivity for phosphate despite the small differences between the two ligands [45]. In the case of GFP, a 'proton wire' is formed from the chromophore hydroxyl group to a water molecule, Ser205, and ultimately Glu222. Infrared absorption analysis indicates that light excitation of GFP leads to protonation of Glu222 and the formation of a LBHB between the chromophore and the water, modulating the emission wavelength [44]. Lastly, as demonstrated by the interactions between KSI and phenolates with various pKa's, the formation of a potential LBHB between the protein and a specific ligand may not necessarily significantly change the binding affinity, but will result in increased enthalpy, which can influence the binding kinetics [78].

5. Summary: Challenges and prospective

Significant progress has been made in our understanding of macromolecular LBHBs, and we have been able to answer several key questions in the LBHB debate, at least partially. New experimental evidence suggests that LBHBs exist in proteins, and they are enthalpically stronger than a standard HB. Additional examples of their potential roles in both enzyme catalysis and protein structures have also been revealed; however, no experimental data have yet to conclusively show how any of these potential LBHBs contribute more to catalysis than a standard HB. The studies on KSI appear to demonstrate that a LBHB in a protein microenvironment is not significantly more stable than an ionic HB in solution, in terms of free energy [78]. Altering the short HB in KSI also did not cause noticeable changes in the reaction rates [83].

Experimental ambiguity remains a key challenge in the study of LBHBs. While crystallography provides the ultimate validation of a delocalized hydrogen, inaccuracy in hydrogen atom positions and crystal artifacts are causes of confusion. Imperfections in perturbation experiments make it difficult to isolate the effect of a single HB. Furthermore, not all experimental techniques are amenable to every protein, leaving gaps in the understanding of a particular macromolecular HB. Compounding these issues is the complexity of enzyme structure and catalysis itself. Multiple short HBs may occur at different stages of the reaction, some at the TS, while others during the formation or collapse of the TS. At a particular reaction coordinate, a network of HBs and other interactions participate in lowering the energy of the reaction intermediate. As the free energy difference between two states is path-independent and is the aggregate result of many competing factors, analyzing the free energy, or enthalpy, of isolated interactions alone may not capture the full kinetic process of the reaction due to the possibility of undercounting, even when there appears to be an agreement between calculations and some experimental data.

Despite these challenges, the comprehensive experimental studies on KSI indicate that it is possible to carry out quantitative investigation of LBHB strength and its catalytic role in proteins. Such experiments provide valuable benchmark data for the development of new computational methods. The convergence of computational and structural analysis of the Class A β-lactamase reaction underscores the value of QM/MM calculations and sub-Angstrom resolution X-ray crystallography. As the number of LBHB examples increases, it

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may be possible to develop a model system where we can apply all available experimental and computational approaches to gain a comprehensive picture of the properties of a particular LBHB. General base catalysis involving proton transfer is one of the areas that can potentially provide useful insights [57,89]. Even though LBHBs in general acid/base catalysis can occur without a net proton transfer [89], the functional groups on the two ends of a proton transfer may have an increased chance of matching pKa's during the transfer process, as well as the necessity to reduce the barrier of the proton transfer. The importance of general base catalysis in both β-lactamase and KSI, and the inconclusive results concerning the short HB in the KSI oxyanion hole, suggest alternative strategies to analyze the functional importance of LBHBs, especially when coupled with theoretical calculations.

While the special role of LBHBs in enzyme catalysis has not been quantitatively established, it is reasonable to ask, if a LBHB is indeed enthalpically stronger than a standard HB in the protein microenvironment, and perturbs the electron density more effectively than other non-covalent forces, why would nature not utilize this unique property? Likewise, other HBs and molecular interactions may all contribute to the stabilization of the TS. The pre-organized features of the enzyme active site not only place these molecular forces in the right place, but also enable them to emerge at the proper time during the reaction. LBHBs may not be the sole driving force of enzyme catalysis, but they may indeed be special and make a stronger contribution than the other non-covalent interactions.

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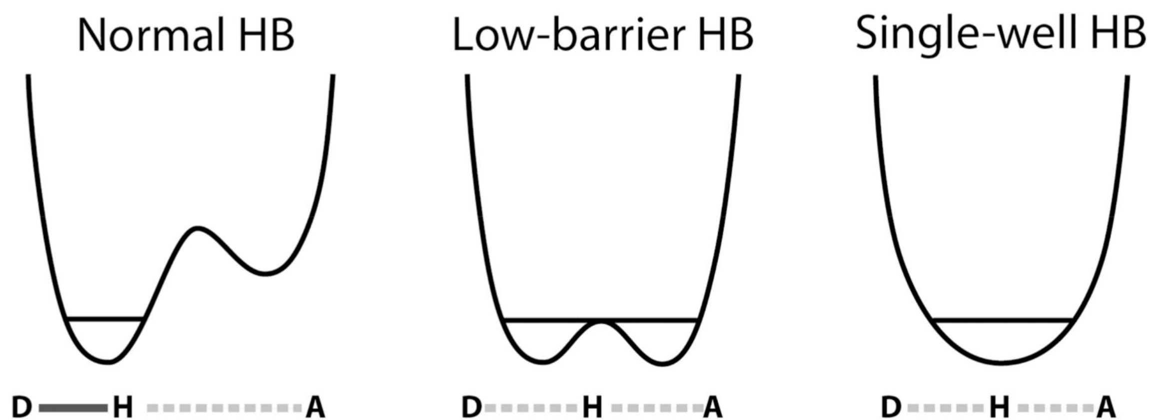

Fig. 1.

Energy potential for a hydrogen atom (H) as a function of its location between the donor (D) and acceptor (A) atom. The horizontal line indicates the first vibrational energy of hydrogen. A normal HB has a localized hydrogen, while the hydrogen in a LBHB or single-well HB is completely delocalized. Intermediate types also exist between normal HBs and LBHBs, with partially delocalized hydrogen atoms. The lengths of normal HBs and the intermediate types can be as short as LBHBs, thus HB length alone cannot be used to distinguish different types of HBs.

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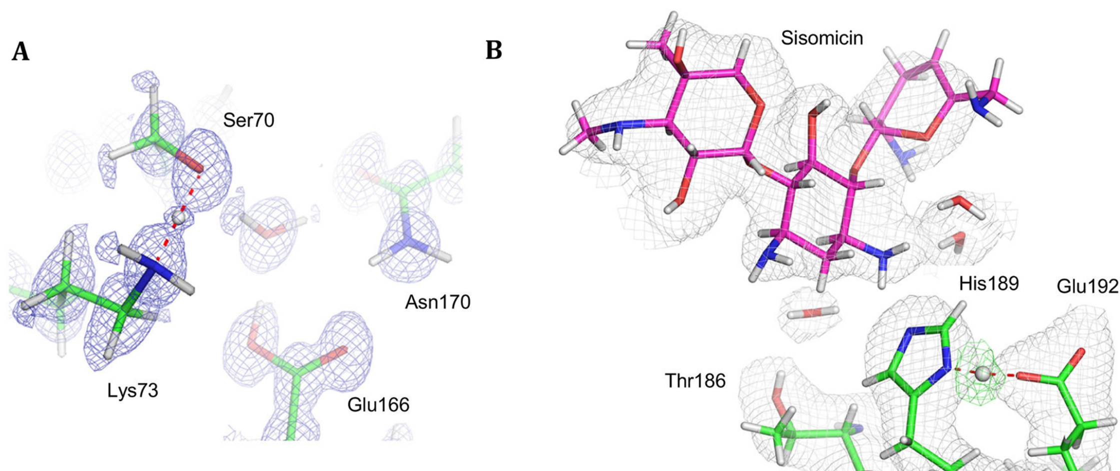

Fig. 2.

Hydrogen/deuterium atoms identified in potential LBHBs. The LBHBs are shown as red dashed lines. The shared hydrogen/deuterium atoms of the LBHBs are shown as white spheres. A) Ser70-Lys73 LBHB in a 0.89 Å resolution X-ray crystal structure of CTX-M β-lactamase (PDB ID, 4UAA, shown in green). The 2Fo-Fc electron density map, shown in blue, is contoured at 0.5 σ. The HB is 2.53 Å in length and the shared hydrogen is at equal distance (1.3 Å) to both Ser70 and Lys73. B) His189-Glu192 LBHB in a 2.20 Å resolution neutron complex crystal structure of aminoglycoside N3-acetyltransferase–VIa (AAC-VIa) (PDB ID, 6BBZ). The protein and ligand (sisomicin) are shown in green and magenta respectively. The 2Fo-Fc nuclear density map and the Fo-Fc deuterium nuclear omit map, adapted from Ref 54 using deposited structure factors, are shown in grey (2 σ) and green (1 σ) respectively. The HB is 2.57 Å in length and the shared deuterium atom is 1.4 and 1.2 Å away from His189 and Glu192 respectively.

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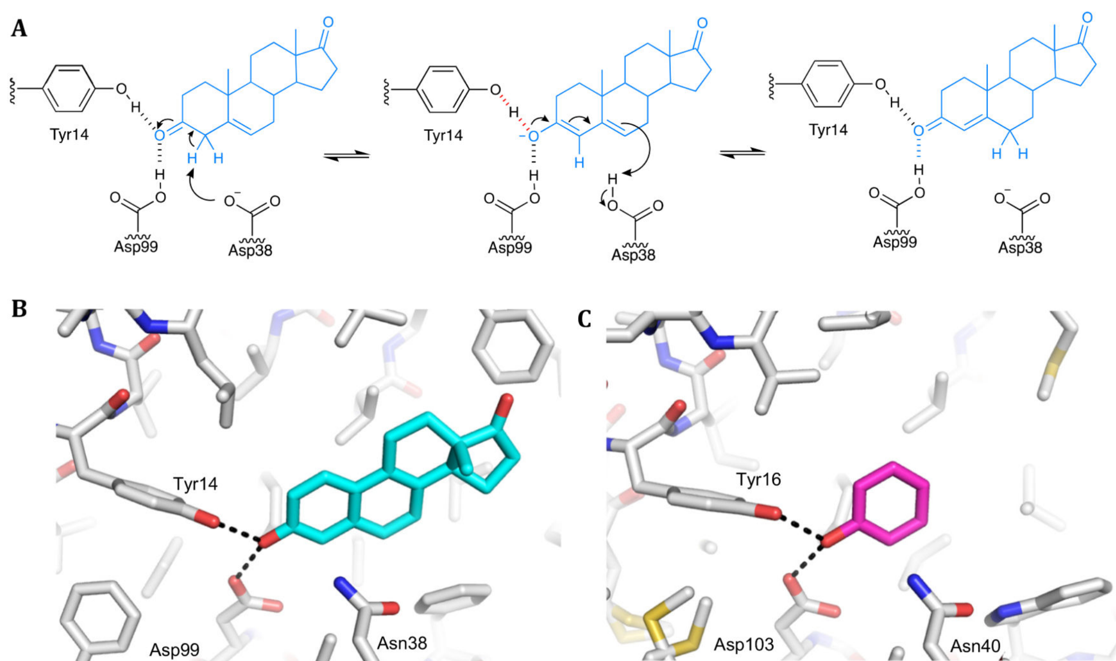

Fig. 3.
Short HBs in KSI active site. A) Catalytic mechanism of KSI. Residue numbering is based on *Comamonas testosteroni* KSI (tKSI). The general base, Asp38, extracts a proton from the substrate, resulting in a negatively charged dienolate intermediate. This intermediate is stabilized by an oxyanion hole formed by Tyr14 and Asp99. The potential LBHB between Tyr14 and the substrate is shown by red dashed lines. The neutral Asp38 then transfers its proton to the intermediate, completing the reaction. B) Complex crystal structure of tKSI D38N mutant with intermediate analog equilenin (PDB ID, 1QJG). The protein and ligand are colored in white and cyan respectively. Tyr14 forms a short HB of 2.5 Å with the ligand. C) Complex crystal structure of *Pseudomonas putida* KSI D40N mutant with phenol (PDB ID, 2PZV). The protein and ligand are colored in white and magenta respectively. Tyr16 also forms a short HB of 2.5 Å with the phenolate oxygen.

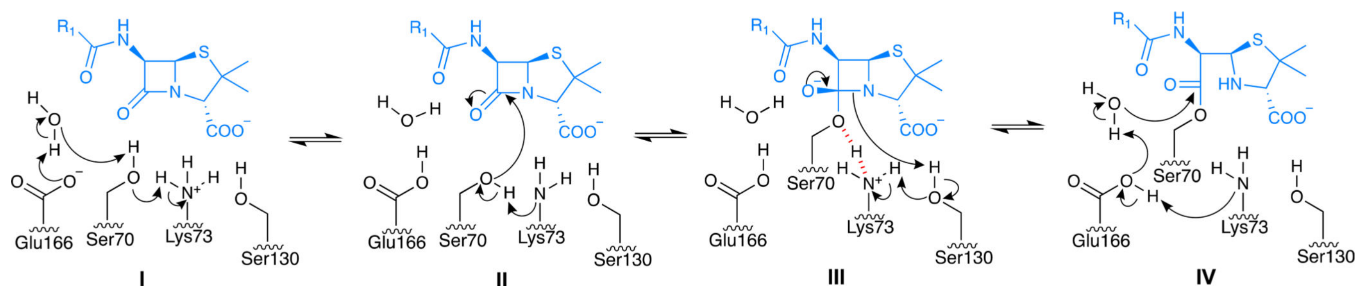

Fig. 4.

Catalytic mechanism of Class A β-lactamase acylation reaction. The binding of the substrate induces protonation state changes in Lys73 and Glu166, either through a series of proton transfers as shown or through a direct proton transfer from Lys73 to Glu166 (stage I). A neutral Lys73 then extracts a proton from Ser70, activating the latter to attack the substrate (stage II). The ensuing TS is stabilized by a LBHB between Ser70 and Lys73, shown by red dashed lines (stage III). During the collapse of the TS, a proton is transferred from Ser130 to the substrate ring nitrogen, followed by a proton transfer from Lys73 to Ser130. This forms the covalent acyl-enzyme intermediate (stage IV). A neutral Lys73 then deprotonates Glu166, enabling the latter to serve as general base and activate the catalytic water to cleave the acyl-enzyme bond. The hydrolyzed product (not shown) is then released from the enzyme active site.

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Table 1

Examples of proposed LBHBs identified by biophysical and computational methods.

Protein	Species	Residues	Technique	PDB ID	Ref	Year
Thermolysin	*Bacillus thermoproteolyticus*	Glu143-(phosphonamidate TSA)[a]	X-Ray	6TMN	[1,2]	1987, 1990
α-Lytic Protease	*Lysobacter enzymogenes*	His57-Asp102 in TSA complex	NMR, (X-Ray*)[b,c]	(2H5D)	[8,9]	1988, (2006)
Carboxypeptidase A	*Bos taurus*	Glu270-(phosphonate TSA)	X-Ray	6CPA, 7CPA, 8CPA	[2,3]	1990, 1991
Triose-Phosphate Isomerase	*Saccharomyces cerevisiae*	His95-(phosphoglycolate TSA carboxylate)	X-Ray, NMR	2YPI	[4,5]	1990, 1991
	Saccharomyces cerevisiae	Glu165-(phosphoglycohydroxmate TSA N)	X-Ray	7TIM	[10]	1991
Aconitase	*Bos taurus*	(Fe bound water) -(inhibitor-carboxylate)	X-Ray	8ACN	[11]	1992
Penicillopepsin	*Penicillium janthinellum*	Asp33-(phosphonate/phosphinate TSA)	X-Ray	1PPM, 1PPL, 1PPK	[12]	1992
Ovotransferrin	*Gallus gallus*	Lys209-Lys301	X-Ray	1NNT	[13]	1993
Mandelate Racemase K166R Mutant	*Pseudomonas aeruginosa*	Glu317-(Substrate carboxylate)	X-Ray	1MDL	[14]	1995
Chymotrypsin	*Bos taurus*	His57-Asp102 in ketone inhibitor complex	NMR, (QM/MM)	–	[6,15–17]	1994, 1997, 1998, (2004)
Citrate Synthase	*Gallus gallus*	Asp375-(TSA inhibitor carboxylate/amide)	X-Ray, NMR	1CSH, 1CSI	[18,19]	1994, 1999
Cytidine Deaminase	*Escherichia coli*	Glu104-(TSA analog OH)	X-Ray	1CTU	[20]	1995
2-Amino-3-Ketobutryate-CoA Ligase	*Bos taurus*	(Unidentified Asp or His)-(PLP cofactor pyridinium NH, or PLP Schiff Base)	NMR	–	[21]	1995
Subtilisin	*Bacillus lentus/ Bacillus licheniformis/ Bacillus amyloliquefaciens*	Asp32-His64 in TSA complex	NMR	–	[22]	1996
-5-3-Ketosteroid Isomerase	*Comamonas testosteroni*	Tyr14-(equilenin oxyanion)	NMR, X-Ray	1QJG	[23,24]	1996, 1999
	Pseudomonas putida	Tyr16-(equilenin oxyanion)	X-Ray	1OH0	[25]	1997
Prolyl Oligopeptidase/ Ogliopeptidase B	*Sus scrofa/Escherichia coli*	His652 in TSA complex, unidentified His in ground state	NMR	–	[26]	1997
Subtilisin	*Bacillus lentus*	Asp32-His64 in apo	X-Ray*	1GCI	[27]	1998
Liver Alcohol Dehydrogenase	*Equus caballus*	Ser48-(Zn bound H_2O)	X-Ray	1QLH	[28]	1999
UDP Galactose 4-Epimerase	*Homo sapiens*	Ser132-(Substrate hydroxyl)	X-Ray	1EK6	[29]	2000
HIV-1 Protease	*Human Immunodeficiency Virus 1*	Asp25-Asp25’	MD, X-Ray, QM/MM	2NPH	[30–32]	2000, 2006
Endothiapepsin	*Cryphonectria parasitica*	Asp32-(inhibitor OH)	X-Ray*, NMR, Neutron*	1GVU, 2VS2	[33,34]	2002, 2008

Protein	Species	Residues	Technique	PDB ID	Ref	Year
Secreted Phospholipase A_2	*Bos tauros*	His48-(phosphonate TSA)	NMR	–	[35]	2003
Photoactive Yellow Protein	*Halorhodospira halophila*	Tyr42-(chromophore OH) Glu46-(chromophore OH)	X-Ray, Neutron*, (QM/MM)	1OTB, 1OT9, 2ZOI, 2ZOH	[36–39]	2004, 2009, (2012, 2014)
Trypsin	*Sus scrofa*	His57-Asp102	(QM-MM)	–	[40]	(2006)
Green Fluorescent Protein (GFP) S65T/H148D Mutant	*Aequorea victoria*	Asp148-(chromophore OH)	X-Ray, (QM/MM)	2DUF (4ZF3)	[41,42]	2007, (2015)
Rhamnogalacturonan Acetylesterase D192N Mutant	*Aspergillus aculeatus*	Asp75-Asp87	X-Ray, NMR	3C1U	[43]	2008
GFP	*Aequorea victoria*	(Chromophore OH)-water	Infrared	–	[44]	2011
Periplasmic Phosphate Binding Protein	*Pseudomonas fluorescens SBW25*	Asp62-(phosphate O2)	X-Ray*	4F1V	[45]	2012
Transketolase	*Homo sapiens*	Glu160-Glu366’	X-Ray*, QM/MM	4KXW	[46,47]	2013, 2019
Pyruvate Oxidase	*Lactobacillus plantarum JDM1*	Glu60-His89’ Glu59’-Thiamine aminopyrimidine	X-Ray*, QM/MM	4KGD	[46,47]	2013, 2019
CTX-M-14 β-Lactamase	*Escherichia coli*	Ser70-Lys73 in non-covalent inhibitor complex	X-Ray, QM/MM	4UAA	[48]	2015
5′-Methylthioadenosine Nucleosidase	*Helicobacter pylori*	Asp198-(product adenine N7)	X-Ray, Neutron*	5CCE	[49]	2016
YajL	*Escherichia coli*	Glu14-Asp23	X-Ray	5SY4	[50]	2017
Aspartate Aminotransferase	*Sus scrofa*	Substrate (Schiff base N_{SB})-(carboxylate group)	X-Ray, Neutron*	5VJZ	[51]	2017
Concanavalin A	*Canavalia ensiformis*	Glu8-Asp28	X-Ray, Neutron*	5WEY	[52]	2017
NS3 Serine Protease of Dengue Virus Type II	*Dengue Virus Type II*	His51-Asp75 in TSA complex (with inhibitor and NS2B)	NMR		[53]	2018
Aminoglycoside N3-Acetyltransferase–VIa	*Enterobacter cloacae*	His189-Glu192	X-Ray, Neutron*	6BBZ	[54]	2018
Aminoglycoside Nucleotidyl Transferase 4′	*Staphylococcus aureus*	Glu52-(neomycin 3’OH)	X-Ray	6UN8	[55]	2020
Transketolase	*Escherichia coli*	Glu411’-Glu160	X-Ray	6TJ8	[56]	2020

[a] TSA, transition state analog.

[b] * Symbol indicates shared hydrogen/deuterium atom identified in the electron/nuclear density map.

[c] Parentheses in Technique, PDB ID, Ref and Year indicate studies against a proposed LBHB.

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