PROTEINS: Structure, Function, and Bioinformatics 55:711–723 (2004)

The Low Barrier Hydrogen Bond (LBHB) Proposal Revisited: The Case of the Asp · · · His Pair in Serine Proteases

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ABSTRACT **The fact that hydrogen bonds (HBs) can provide major stabilization to transition states (TSs) of enzymatic reactions is well known. However, the nature of HB stabilization has been the subject of a significant controversy. It is not entirely clear if this stabilization is associated with electrostatic effects of preorganized dipoles or with delocalized resonance effects of the so-called low barrier hydrogen bond (LBHB). One of the best test cases for the LBHB proposal is the complex of chymotrypsin and trifluoromethyl ketone (TFK). It has been argued that the pK_a shift in this system provides an experimental evidence for the LBHB proposal. However, this argument could not be resolved by experimental studies. Here we explore the nature of the Asp102–His57 pair in the chymotrypsin-TFK complex by a systematic computational and conceptual study. We start by defining the LBHB proposal in a unique energy-based way. We show that a consistent analysis must involve a description in terms of the energy of the two resonance structures and their mixing. It is clarified that LBHBs cannot be defined according to strength or distance, because ionic HBs can also be strong and short. Similarly, NMR chemical shifts and fractionation factors cannot be used to identify LBHBs in a conclusive way. It is also clarified that HBs with a significant asymmetry cannot be classified as LBHBs, because this contradicts the assumption of equal pK_a of the donor and acceptor. Thus, the main issue is the ΔpK_a and the corresponding energy difference. With this definition in mind, we calculate the free energy surface of proton transfer in this pair and evaluate the energetics of the different ionization states of this system. The calculations are done by both the semimacroscopic version of the protein dipoles Langevin dipoles (PDLD/S-LRA) model and by the empirical valence bond (EVB) method. The calculations establish that the LBHB proposal is not valid in the chymotrypsin-TFK complex and in other serine proteases. Although previous theoretical studies reached similar conclusion, this is the first time that the same set of free energy calculations reproduce all the known pK_a values and pK_a changes in the system, while evaluating the energetics and covalent character of the His–Asp system. The present study provides a support to the idea that enzymes work by creating a preorganized polar environment. Proteins 2004;55:711–723.**

INTRODUCTION

The elucidation of the molecular origin of enzyme catalysis is a problem of major fundamental and practical importance.[1,2] Although the issue of reactant state destabilization (RSD) versus transition state stabilization (TSS) is still actively debated,[3] there is growing agreement about some elements of the TSS proposal. In particular, there are clear evidences that hydrogen bonds (HBs) play a major role in TSS (e.g., see Refs. 4 and 5). Nevertheless, the catalytic role of HBs has been the subject of different proposals and significant controversy. Here we will focus on the so-called low barrier hydrogen bond (LBHB) proposal and its relationship to other HB catalytic proposals.

The first concrete support to the idea that HBs contribute to enzyme catalysis can be traced back to the identification of the oxyanion hole in subtilisin.[6] However, this structural observation did not involve any estimate of the relevant catalytic energy. Subsequent theoretical studies[5,7,8] have established the idea that the overall electrostatic effect of preorganized hydrogen bonds contributes in a major way to enzyme catalysis. These theoretical predictions were confirmed by mutation experiments, showing clearly that a single hydrogen bond can contribute ~5 kcal/mol to an ionic transition state.[4,9] The results of some specific mutation experiments were subsequently reproduced by free energy perturbation/umbrella sampling (FEP/US) calculations.[7]

After the experimental demonstration of TS stabilization by HBs, it was proposed by several workers that HBs stabilize TSs in a special non-electrostatic covalent way, which was termed low barrier hydrogen bond (LBHB).[10–12] The LBHB proposal has suggested that catalytic HBs involve a flat minimum rather than a double minimum. Unfortunately, this suggestion (which is sometimes true) does not allow one to distinguish the LBHB proposal from the previous proposal of ionic HBs (and thus, does not

Grant sponsor: National Institutes of Health; Grant number: GM24492.

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Received 24 September 2003; Accepted 4 December 2003

Published online 1 April 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/prot.20096

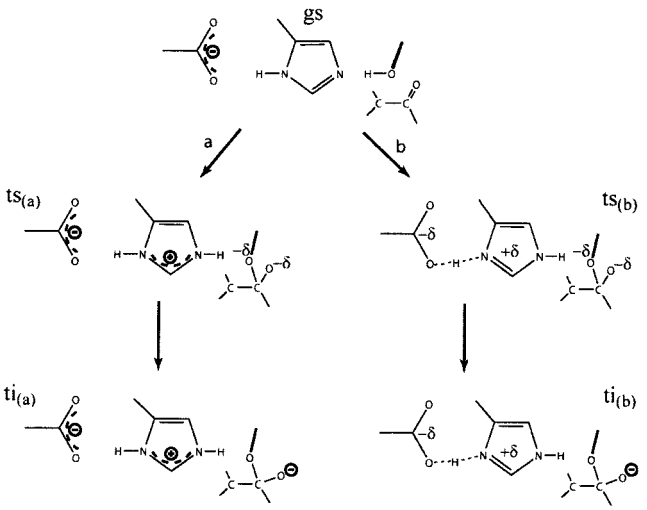

Fig. 1. Two alternative mechanisms for the catalytic reaction of serine proteases. Branch (**a**) corresponds to the electrostatic catalysis mechanism, whereas branch (**b**) corresponds to the proton sharing mechanism (the LBHB mechanism). gs, ts, and ti denote the ground state, transition state, and tetrahedral intermediate, respectively.

provide a testable definition). This issue will be addressed in more detail in Defining the Problem.

An excellent test case for the analysis of the LBHB proposal is offered by the catalytic triad of serine proteases (see Ref. 13 for the definition of the relevant system). The reaction of this system can be described schematically by the two alternative mechanisms shown in Figure 1 (see also Ref. 1). One option is the ionic mechanism [branch (a)], and the other option, [branch (b)], is a TSS mechanism where the TS is stabilized by a delocalized [$Asp^{-\delta}$ $His^{+\delta}$]LBHB system (see below for a more precise definition). Frey and coworkers[11] put forward this system as a prime example of LBHB catalysis. As with all other LBHB proposals, they assumed that the pK_a difference between His 57 and Asp 102 at the TS of the hydrolysis reaction is close to zero[11] and that the barrier for proton transfer (PT) is small. This means that the Asp-His system can be described as [$Asp^{-0.5} \cdots H \cdots His^{+0.5}$] at the TS or in the presence of a TS analog (TSA); attempts to modify this proposal to an assertion that the LBHB corresponds to asymmetric charge distribution will be shown to be inconsistent with the assumption of equal pK_a values and with the charge distribution drawn by the LBHB proponents (see also below). However, Warshel and coworkers[14,15] argued, on the basis of early calculations,[13,16] that in the protein the pK_a of Asp102 is lower than that of His57, and thus, the proton must be on His57. This argument is supported by NMR studies.[17,18] It was also argued[14] that the LBHB proposal implies that the ionic state is destabilized in the protein and that this corresponds to an anticatalytic effect. Nevertheless, Cassidy et al.[19] put forward a new argument, while still overlooking the above points, and in particular the pK_a issue. That is, these workers (see also Ref. 20) considered the highly relevant system of chymotrypsin with a TFK (trifluoromethyl ketone) TSA (which will be sometimes referred to here as t^- to designate its nature as a negatively charged bound tetrahedral intermediate) where the pK_a of His57 is shifted from ~7 to −11. They then argued that the pK_a shift is due to a binding induced steric strain between Asp102 and His57, whose release supposedly leads to an LBHB. As pointed out before,[3] this proposal is problematic. First, there is no obvious reason (or evidence) for binding induced steric strain between Asp102 and His57. Second, it is very likely that a negatively charged TS and also the complex with TFK should increase the ΔpK_a of His57 (because the protonated histidine is positively charged and because a positive charge is stabilized by a negative charge), and thus increase the pK_a difference between Asp102 and His57 and reduce (rather than increase) the LBHB contribution. Now, although the above simple electrostatic considerations are inconsistent with the LBHB proposal of Ref. 19, we find the TFK system provides one of the best benchmarks for the examination of the LBHB proposal.

This work considers the LBHB proposal but focuses on serine proteases in general and on the TFK system in particular. The next section defines the LBHB proposal in a unique way by using a valence bond description of the relevant resonance structures. This section also discusses the problem with some alternative definitions. The theoretical Methods section describes the theoretical methods that are being used to examine the LBHB proposal in the Asp102–His57 pair in chymotrypsin and in the chymotrypsin/TFK complex. The Results section describes the results obtained by the semimacroscopic version of the protein dipoles Langevin dipoles method (the PDLD/S-LRA approach) and by the microscopic empirical valence bond (EVB) model. The conclusions that emerged from the calculations and from other general considerations are discussed in the Discussion section.

DEFINING THE PROBLEM

HBs Can Be Defined in a Consistent Way by VB Free Energy Diagrams

To distinguish between ionic HBs and LBHBs, it is essential to first define the LBHB proposal in a clear way, which reflects the energetics of the system and can be classified as a catalytic or anticatalytic effect. At present, the best way to define the LBHB proposal is to use the Valence-Bond (VB) representation. This representation can be treated in a simplified two-state version of the three-state model of Coulson and Danielsson,[21,22] augmented by considering the EVB solvent effect.[1] Here we consider as an example the [$X^-H\text{-}Y \rightleftharpoons X\text{-}HY^-$] system, but the same considerations will be applicable to the [$X^-H\text{-}B^+ \rightleftharpoons X\text{-}HB$] system. At any rate, in the two-state VB representation, we can describe the total wave function by

$$\Psi = C_1\Phi_1 + C_2\Phi_2 \qquad (1)$$

where Φ_1 = [$X^-H\text{-}Y$] and Φ_2 = [$X\text{-}H\ Y^-$] are the diabatic wave functions whose energies are E_1 and E_2, respectively. The coefficients C_1 and C_2 and the ground state free energy, E_g, are obtained by solving the two-state VB secular equation in its EVB representation, where Φ_1 and

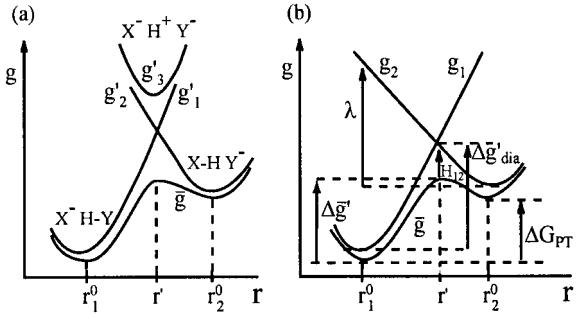

Fig. 2. **a:** A three-state VB system (Φ_1 = [X^- H-Y], Φ_2 = [X-H Y^-], andΦ_3 = [X^- H^+ Y^-]) with the corresponding diabatic free energies g′$_1$, g′$_2$, and g′$_3$. **b:** The projection of the three-state model on a two-state VB representation. The mixing of the two surfaces (whose free energy surfaces are g_1 and g_2) leads to a ground state surface with a free energy function $\bar{g}$. When the donor and the acceptor are held at a distance R, the stabilization resulting from the mixing of the two "impure" states is given by H_{12} (R,r), and its value at r = r′ is denoted simply by H_{12}. The coordinate r corresponds to proton movement at fixed R, where r_1^0 and r_2^0 are the equilibrium coordinates of X-H and H-Y, respectively. Δg′ and $\Delta g'_{dia}$ are the adiabatic and diabatic activation barriers (see Ref. 14 for more quantitative discussion).

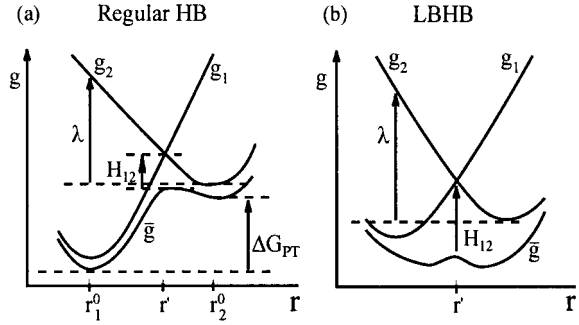

Fig. 3. **a:** A two-state VB model for an ionic hydrogen bonded system (see Ref. 14). The free energies g_1 and g_2 correspond to the states [X^- H-Y] and [X-H Y^-]. The ground state surface E_g (with a corresponding free energy surface &gmacr;) is obtained from the mixing of the two states. The donor and the acceptor are held at a distance R. The equilibrium distances for isolated X-H and H-Y fragments are designated by r_1^0 and r_2^0 λ and ΔG_{PT} designate the reorganization energy and proton transfer energies, respectively. **b:** A two-state VB model in the LBHB limit. In this case, $\Delta G_{PT} \approx 0$ and $H_{12} \geq \lambda/4$.

$\Phi_2$are assumed to be orthogonal wave functions whose off diagonal resonance integral is the mixing term H_{12} (see Refs. 1 and 14 for this description). Now we can approximate the adiabatic ground state free energy by[14]

$$\bar{g}(r_1, R) \cong [(g_1 + g_2) - ((g_1 - g_2)^2 + 4H_{12}^2)^{1/2}]/2 \quad (2)$$

where r_1 is the H-Y bond length and R is the X–Y distance, whereas g_1 and g_2 are the diabatic free energy functionals that correspond to the diabatic potential surfaces E_1 and E_2, respectively. This expression is obtained by converting the EVB ground state potential energy, E_g, to the corresponding ground state free energy functional, $\bar{g}$, by the EVB umbrella sampling approach. We can also approximate the adiabatic free energy barrier by

$$\Delta\bar{g}' = \Delta g'_{\rm dia} - H_{12} + H_{12}^2/(\lambda + \Delta G_{\rm PT}) \quad (3)$$

where $\Delta\bar{g}'_{dia}$ is the diabatic free energy barrier (see Fig. 2) obtained from the intersection of g_1 and g_2 and where the second and third terms reflect the differences between the diabatic and adiabatic energy at r' and r_1^0. Equation 3 allows us to immediately define the limits of a LBHB and ionic HB. That is, because $\Delta g'_{dia}$ can be approximated by the Marcus formula and be expressed as $\Delta g'_{dia} = (\lambda + \Delta G_{\rm PT})^2/4\lambda$, we obtain $\Delta g'_{dia} \simeq \lambda/4$ when $\Delta G_{\rm PT} \simeq 0$. Thus, we will have a single minimum or a very small barrier at $r_1 \simeq r'$for $\Delta g'_{dia} \simeq |H_{12}|$ and $\Delta G_{\rm PT} \simeq 0$ [see Fig. 3(b)]. On the other hand, when $\lambda/4 \gg |H_{12}|$ we will have a double minima system, which cannot be classified as an LBHB. We also note that the LBHB proponents distinguished between a single minimum and a small barrier at $r_1 \simeq r'$, but this does not change *any* of our conclusions with regards to the interplay between λ and H_{12}. The situation becomes much clearer when $|\Delta G_{\rm PT}| > 0$. In this case, we have an ionic HB (Φ_1 or Φ_2 depending on the sign of $\Delta G_{\rm PT}$) and we cannot describe the system as an LBHB.

The transition between the LBHB and HB limits can be further quantified by considering the behavior of $\Delta\bar{g}'$ and asking when this barrier becomes small. This can be formulated by following Ref. 14 and defining a parameter θ by the relationship

$$\theta = H_{12}/\lfloor \Delta g'_{\rm dia} + H_{12}^2/(\lambda + \Delta G_{\rm PT}) \rfloor \quad (4)$$

This equation satisfies the relationship $\Delta\bar{g}' = H_{12}(1 - \theta)/\theta$ where $\Delta\bar{g}'$ is the adiabatic free energy barrier of Eq. 2. Now, when $\theta \geq 1$ we have $\Delta\bar{g}' \leq \theta$, and the system can be classified as an LBHB.

This shows how the interplay between the covalent mixing H_{12} and the electrostatic (solvation) effects, which are reflected by λ and $\Delta G_{\rm PT}$, determines the nature of ionic HBs. It is also important to note that $\Delta G_{\rm PT}$ is linearly correlated with the pK_a difference between the donor and acceptor; thus, we have a clear relationship between the LBHB character and $\Delta G_{\rm PT}$. Now, when $\theta \leq 1$ and when the minimum of the adiabatic ground state is near r_1^0, we can use perturbation theory and write

$$C_2^2(r \simeq r_1^0) = (H_{12}/(g_2 - g_1))^2_{r \simeq r_1^0}$$
$$= (H_{12}/[\lambda + \Delta G_{\rm PT} + H_{12}^2/(\lambda + \Delta G_{\rm PT})])^2 \quad (5)$$

Note, that $g_2 - g_1$, is rigorously equal to $E_2 - E_1$.[23] The magnitude of C_2^2 can tell us how much delocalization we still have in the given HB. At any rate, we established here that the existence of an LBHB is defined in terms of the competition between H_{12} and $(\lambda + \Delta G_{\rm PT})$. In other words, we are dealing here with a competition between the localized [X-H Y^-], [X^-H-Y] pictured and delocalized [$X^{-0.5} \cdots H \cdots Y^{-0.5}$] pictures. In the gas phase, the delocalized picture tends to dominate, whereas in solution the localized picture is more important.

With the above limiting cases in mind, we can ask what is new in the LBHB proposal. Obviously, the idea that HBs, which are preorganized to stabilize ionic TSs, contribute to catalysis is not new (see above). Thus, the only new

element in the LBHB proposal is the idea that the covalent delocalized character, which leads to the single energy minimum, is the origin of the catalytic effect. In this respect, it should be clear that HBs in solution have a significant covalent character (for an early demonstration see Ref. 24). Furthermore, for the LBHB proposal to be valid, the covalent character must be larger in the enzyme than in solution, and the corresponding difference must be the source of the HB catalytic effect. Obviously, these issues cannot be examined without evaluating the relevant energies.

At this point, it is important to clarify that the entire issue of the validity of the LBHB proposal is related to the interaction between the environment and the VB states of the given ionic HB (in the gas phase, we frequently have LBHBs). The LBHB proponents, who originally assigned to LBHBs in enzymes the enormous energy of ~20 kcal/mol, of gas phase LBHB (e.g., see Ref. 10), argued that the enzyme environment is nonpolar and thus should lead presumably to gas phase-like LBHB. However, such desolvation arguments are not useful without actual calculations of the relevant polarity and the corresponding solvation effect (in fact, all consistent studies show that enzyme sites are very polar (e.g., see Ref. 1). Performing such calculations in a reliable way is the best way to examine the LBHB proposal.

Defining LBHBs According to Strength and Classifying Asymmetric HBs as LBHBs is Inconsistent With the LBHB Proposal

As mentioned above, we believe that the best way to describe HBs is to use the VB picture. There are other definitions, however, that should be considered here. For example, Hibbert and Emsley[25] classified HBs according to what they called weak, intermediate, and strong HBs. Now, this and any alternative description of HBs can be recovered by the VB description. Furthermore, and more importantly, the notion of strength of HBs, which is reasonable when one deals with HBs in a single phase (e.g., gas or solution), becomes extremely problematic when one deals with HBs in proteins. Here what counts is the energy relative to the corresponding energy in water (stability rather than force). The best way to see this fact is to realize that an ionic HB is very strong in the gas phase but much less stable (much higher energy) than in water (see Ref. 14 for a clear demonstration of this issue). Once we avoid the ill-defined concept of "strength," we are back to the above VB definition of the relative energy of the two VB states.

At this point, it is also useful to consider the idea that the LBHB proposal also applies to asymmetric HBs (e.g., see Ref. 20). One can perhaps trace this proposal to the statement in Ref. 11 that when the fractional charge (δ) on the donor can be between 50% to almost 100%, we can have an LBHB. Unfortunately, if we allow the fractional charge to be close to 100%, we clearly cannot have a LBHB proposal because this contradicts the assumption of equal pK_a ($\Delta G_{PT} \simeq 0$), which is shared by all the LBHB proponents including Ref. 11. Having $\delta \sim 1$ corresponds exactly to the localized ionic HB concept, which means that assigning such a system as a LBHB cannot be a new proposal (see above). Apparently, the suggestion that an asymmetric HB is compatible with the LBHB proposal is simply inconsistent with the requirement of having a new proposal or having $\Delta pK_a \sim 0$. In other words, the common case of asymmetric single minimum ionic HBs is not a LBHB but a clear case where the ΔpK_a is large. Note in this respect that the idea that LBHB may involve asymmetric charge distribution is in contrast with the molecular figures presented by the LBHB proponents (e.g., Scheme 1 in Ref. 26).

It is also important to point out that the LBHB cannot be defined by such terms as short strong HB (SSHB), because an ionic HB can also be short. Furthermore, in contrast to statements (e.g., Ref. 20) that the LBHB proposal does not imply that the proton is found in equal distance from the donor and acceptor, this is exactly the requirement from a consistently defined LBHB model. That is, if we have a large ΔG_{PT}, we can have an asymmetric HB with [X^- H-Y] as a dominant form so that the proton will be attached to Y. Because this will be clearly an ionic HB, we conclude that the identification of LBHB with a single minimum system is *only* valid when the minimum is at the center of the X–Y vector (the same is true even if we have a small barrier at the center).

The LBHB proposal has been sometimes misrepresented by implying that the LBHB is a strong HB while presumably the HB considered in electrostatic proposals is a weak, neutral HB.[27] Similarly, it was implied repeatedly (e.g., Ref. 28) that strong hydrogen bond stabilization must be associated with the LBHB proposal, in contrast with the presumably weak HB effect in other enzymatic proposals. However, all the electrostatic preorganization proposals have been associated with large (3–5 kcal/mol) contributions of preorganized ionic HBs.[1,5,7] Thus, in clear contrast to the implication of Ref. 28, the strength (or more precisely the energy) contribution of a hydrogen bond cannot be used as a definition of the LBHB proposal. Again, as stated above, only the degree of covalent mixing, or the relative energy of the two relevant VB states, can be used to designate the LBHB idea as a new catalytic proposal.

As much as the definition of the LBHB proposal is concerned, it is important to address the repeated attempts to use experimental observations as operational definitions of this proposal (e.g., Refs.12 and 26). Apparently, such a definition confuses the interpretation of experiments with experimental facts. As will be shown in the Discussion section, most experimental based definitions of the LBHB proposal are equally consistent with the existence of an ionic HB. Several experiments (e.g., studies of the N-H distance) are much more consistent with the ionic HB than the LBHB picture. However, the most crucial issue is the relative energy of the VB states or the ΔpK_a (or ΔG_{PT}) in the protein active site at the TS. Now, $\Delta pK_a > 0$, but this is inconclusive because we have no experimental assignment of all the relevant protonation

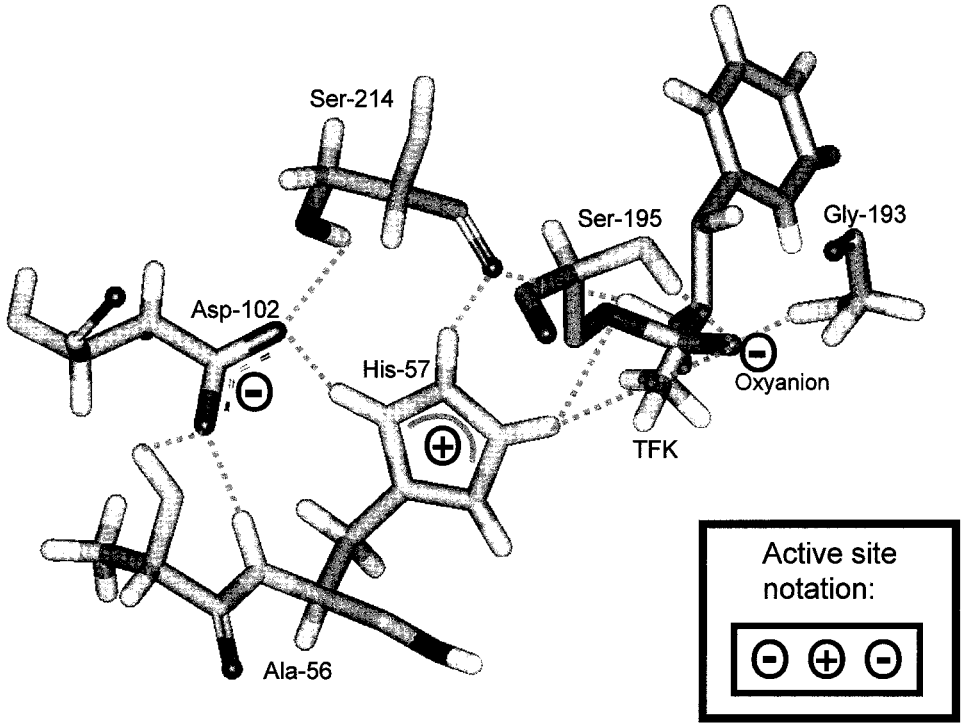

Fig. 4. The hydrogen-bonding network created in the active site of the chymotrypsin/TFK TS analog. The $(- + -)$ active site notation is defined here by Asp102, His57, and the oxyanion of the bound TFK.

states (see below) of the states involved. Thus, it is crucial to use theoretical calculations to resolve the LBHB issue.

THEORETICAL METHODS

The present work examines the LBHB proposal by considering the covalent complex of γ-chymotrypsin/TFK,[19] which is described in Figure 4. The X-ray coordinates of this system were taken from PDB 6GCH[29] and used as the starting point of the simulations. The structure and charge distribution of the complex are close to that of the TS of the reaction of serine proteases; thus, the complex serves as an excellent system for exploring the LBHB proposal. Now, to analyze the LBHB proposal in a conclusive way, we use complementary methods, the PDLD/S-LRA method for electrostatic calculations and the EVB method for direct calculation of the free energy surfaces for the PT process. Both of these methods are described briefly below.

The PDLD/S-LRA Method

To calculate electrostatic energies in general and the relevant pK_a values in particular we use the PDLD/S-LRA method.[30,31] This method evaluates the change in solvation free energies upon transfer of a given group or groups to the protein by using the effective potential.[30]

$$\Delta U^{w\to p}_{sol,i} = [-\Delta G^{w}_{sol,i} + \Delta G^{w}_{sol,p}(q = q_i) - \Delta G^{w}_{sol,p}(q = 0)] \times \left(\frac{1}{\varepsilon_p} - \frac{1}{\varepsilon_w}\right) + \Delta U_{q\mu}\frac{1}{\varepsilon_p} \quad (6)$$

where $\Delta G^{w}_{sol,i}$ is the free energy of solvation of the ith ionizable group in water (the self-energy in water), $\Delta G^{w}_{sol,p}$ $(q = q_i)$ and $\Delta G^{w}_{sol,p}$ $(q = 0)$ are the free energies of solvation of the entire protein in water with atomic charges present on the particular group ("charged state") and with atomic charges on the group set to zero ("uncharged state"), respectively. The $\Delta G^{w}_{sol,p}$ $(q = 0)$ term approximates the solvation of the protein in the case in which the ionizable group is not ionized. $\Delta U_{q,u}$ is the vacuum interaction between the atomic charges on the ionizable group and the permanent dipoles of the protein (represented by atomic charges), ϵ_w is the dielectric constant of water, and ϵ_p is the dielectric constant of the protein, which is basically a semimacroscopic scaling factor that accounts for the interactions that are not considered explicitly. This factor is quite different from the actual protein dielectric constant (see Ref. 32).

To capture the physics of the reorganization of the protein dipoles in the charging process, it is necessary to relax the protein structure in the relevant charged and uncharged states. Moreover, for accurate free-energy differences, several protein configurations should be averaged. The configurational space can be adequately sampled by using Monte Carlo or molecular dynamics (MD) techniques.[33] In this study, we use an MD approach in the LRA framework.[30,31] This approach approximates the free energy associated with a transformation between two charged states by averaging the potential difference between the initial and final states over trajectories propagated on these two states, respectively. Using the PDLD/S free energy that corresponds to a single protein structure as an

effective potential in the PDLD/S-LRA method, the free energy of solvation is given by

$$\Delta G_{\mathrm{sol,i}}^{\mathrm{w}\rightarrow\mathrm{p}} = \frac{1}{2}\left[\langle \Delta U_{\mathrm{sol,i}}^{\mathrm{w}\rightarrow\mathrm{p}}\rangle_{q=q_{\mathrm{i}}} + \langle \Delta U_{\mathrm{sol,i}}^{\mathrm{w}\rightarrow\mathrm{p}}\rangle_{q=0}\right] \qquad (7)$$

where the $\Delta U_{sol,i}^{w\rightarrow p}$ is the PDLD/S effective potential of Eq. 6; the $\langle\ \rangle_{q=q_{\mathrm{i}}}$ and $\langle\ \rangle_{q=0}$ terms designate an average over protein configurations generated in the charged and uncharged state of the given group, respectively. Although this approach takes into account the reorganization of the environment explicitly, it may not fully account for some effects such as the complete water penetration and protein reorganization. These factors and the effect of induced dipoles are implicitly included in the model, which lead to the use of ϵ_{p} in the PDLD/S model.

The basic PDLD/S-LRA calculations are performed with all the protein groups (except some active site residues) in their neutral form. The effect of ionizing these groups is evaluated macroscopically by finding their ionization state in a self-consistent way[31] and then evaluating the effect of these groups by using a distance-dependent dielectric constant.

As described and justified elsewhere,[30,32] with PDLD/S-LRA solvation free energies, we can evaluate the apparent pK_a of any given group. This is done by using[30]

$$\mathrm{pK}_{\mathrm{app,i}}^{\mathrm{p}} = \mathrm{pK}_{\mathrm{a,i}}^{\mathrm{w}} - \frac{\bar{q}_{\mathrm{i}}}{2.3RT}\Delta\Delta G_{\mathrm{solv,i}}^{\mathrm{w}\rightarrow\mathrm{p}} + \Delta\mathrm{pK}_{\mathrm{a,i}}^{\mathrm{charges}} \qquad (8)$$

where $\bar{q}_{\mathrm{i}}$ is the charge of the ionized form of the given residue; for acidic groups, we have $\bar{q}\mathrm{i} = -1$ ($q(\mathrm{AH}) = 0$, $q(\mathrm{A}^-) = -1$) and for basic groups we have $\bar{q}_{\mathrm{i}} = +1$ ($q(\mathrm{AH}) = +1$, $q(\mathrm{A}^-) = 0$). The $\Delta\mathrm{pK}_{a,i}^{charges}$ reflects the effects of all other ionized groups on the ith group. This effect is evaluated self-consistently by using a distance-dependent dielectric constant for charge–charge interactions (e.g., see Ref. 32). Because $\mathrm{pK}_{a,i}^{w}$ can be determined experimentally, one needs to focus only on the second term in Eq. 8. Similar treatment is used for the energy of ion pairs.

The PDLD/S-LRA calculations involve as usual (e.g., see Ref. 31) two steps: first running MD to generate protein configurations for the charged and uncharged states and then averaging the PDLD/S results for the generated configurations. The MD runs are performed with three models designated here as A, B, and C. Model A uses the nonpolarizable ENZYMIX force field,[30] model B uses the polarizable ENZYMIX force field,[30] and model C uses a polarizable force field where the energy of the induced dipoles is considered explicitly, but the forces are obtained without the induced dipole force (instead, the residual charges are scaled down.[31]). All the PDLD/S-LRA calculations are performed by the automated procedure of the MOLARIS program,[30] in which we generate typically 20 configurations for the charged and uncharged state, using MD simulations of 1 ps, with a 1-fs timestep, for each configuration.

EVB Calculations

The EVB approach[1,24] provides what is at present perhaps the most reliable way of evaluating the proton transfer (PT) profile. It is calibrated with the experimental information about the reference reaction in solution and it involves complete converging free energy perturbation simulations that samples consistently the protein configurational states. Furthermore, this approach takes into account non-equilibrium solvation effects that are not accounted for by regular PMF calculations.[34] Because the EVB approach was described extensively elsewhere, we mention here only several key points.

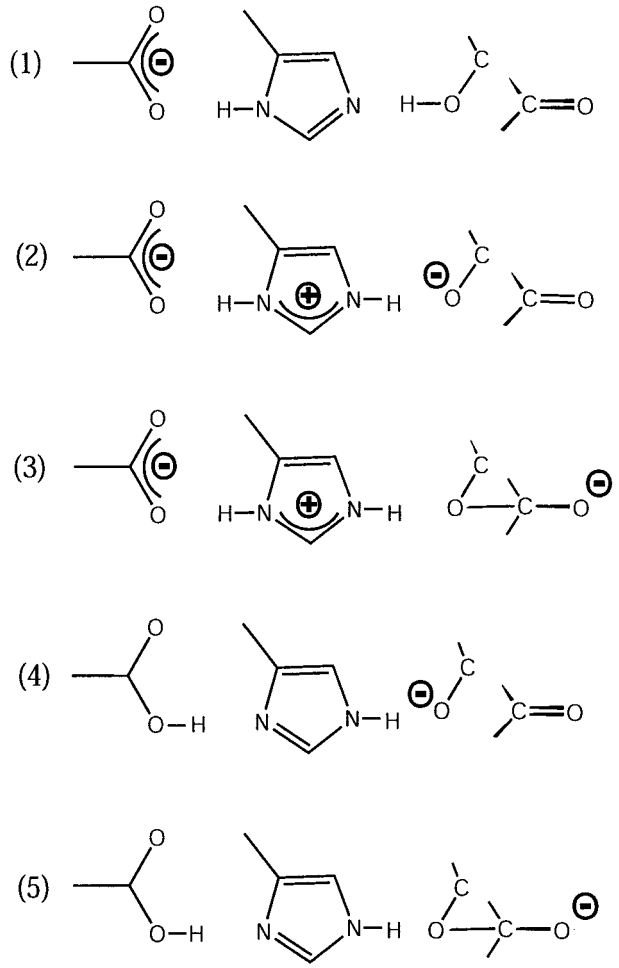

Fig. 5. The VB states used in the present EVB treatment of the catalysis reaction of serine protease.

The EVB surface evaluated in the present study is basically very similar to the surface involved in our early studies,[7,13,16] and the key resonance structures are shown in Figure 5. The main difference is the calibration for the TFK system forced the tetrahedral intermediate (state 3 and/or state 5) to be a stable intermediate.

The LBHB issue is explored by examining the potential surface and mixing of states 3 and 5 as a function of PT from His57 to Asp102. The key point in terms of the reliability of the present work is the calibration of the energetics of the PT processes in solution using a reliable experimental estimate. This is done as in our previous studies using the relationship[13,16]

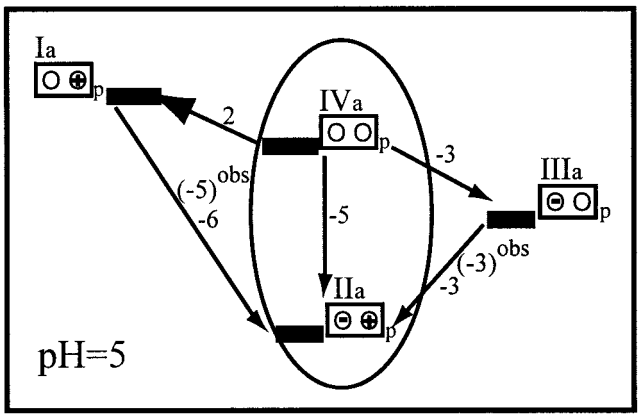

Fig. 6. The energetics of the different ionization states of the Asp102–His57 system in the free enzyme (with a neutral Ser195). The left and right ionization states correspond to Asp102 and His57, respectively. The energies of the transitions between the different states are given in kcal/mol.

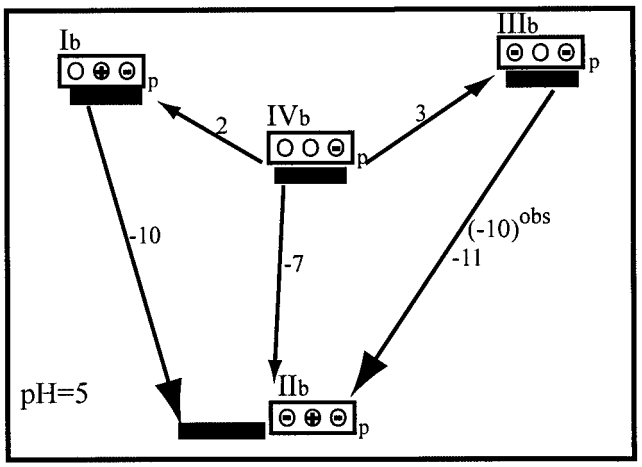

Fig. 7. The energetics of the different ionization states of the Asp102–His57 system in the presence of the bound TFK. The three ionization states correspond to the notation of Figure 4 (in the order Asp102, His57, t^-, from left to right). The energies of the transitions between the different states are given in kcal/mol.

$$\Delta G^{w}_{PT} = 2.3RT[pK^{w}_{a}(Asp) - pK^{w}_{a}(His)] + V_{QQ}/\epsilon_{eff} \quad (9)$$

where the pK^{w}_{a} values are the pK_a values in water for Asp and His, 3.9 and 6.5, respectively. V_{QQ} is the interaction between the residual charges on the given fragments. $\epsilon_{eff} = 40$ is a reasonable estimate for ϵ_{eff} in water when the two charges are at a close distance, as in the case of our ion pairs. Now, forcing the EVB free energy surface for the solution reaction to reproduce the solution energetics (obtained by Eq. 9) guarantees the reliability of the EVB calculations in the protein.

All the EVB calculations were done with the ENZYMIX module of the MOLARIS program.[30] The calculations involve the surface constraint all atom solvent (SCAAS) spherical boundary conditions[35] and the local reaction field (LRF) long-range treatment.[36] The free energy simulations were done with 21 windows of 10 ps each. The simulations were done with timesteps of 1 fs at 300 K.

RESULTS

As stated above, our aim is to examine the LBHB proposal by two complementary methods: the PDLDS-LRA and the EVB approaches. The results of these approaches are summarized below, emphasizing the relationship of the calculations to relevant experimental observations.

Evaluation of the Energetics of the Catalytic Triad by the PDLD/S-LRA Approach

To address the LBHB proposal in a conclusive way, it is essential to validate the calculations by relating them to the experimental pK_a values in the protein. This task is far from trivial because the system involved different possible protonation states (see Figs. 6 and 7), and the energetics of some of these states cannot be evaluated by direct experiments.

To analyze the energetics of the different protonation states, we used the PDLD/S-LRA approach described in Theoretical Methods. The calculations involve both direct pK_a calculations (Table I) and calculations of the free energies of different configurations (Table II) using the relationship[37]

$$\Delta G^{p} = \Delta G^{w} + \Delta\Delta G^{w\rightarrow p}_{sol} \quad (10)$$

where ΔG^{p} and ΔG^{w} are the free energies in the protein and in solution, and $\Delta\Delta G^{w\rightarrow p}_{sol}$ is the change of solvation free energy of the specific configuration upon transfer from water to the protein active site. In cases of steps that involve ionization of a single group we used the relationship

$$\Delta G^{w} = -2.3RT\bar{q}_i(pK^{w}_{a,i} - pH) \quad (11)$$

where $\bar{q}_i$ is the charge of the ith group (−1 or +1), $pK^{w}_{a,i}$ is the pK_a of the given residue in water (3.9 and 6.5 for Asp and His, respectively). The pH term was taken as 5 in all calculations.

The results of this analysis are also summarized in Figures 6 and 7 and Tables I and II. As seen from the figures and the tables, the ion pair state is more stable than the nonpolar pair state by ~6–8 kcal/mol in the presence of the TS analog and about ~5–7 kcal/mol in the absence of the TS analog. The same calculations reproduced the observed change in the pK_a of His 57 (~12 and ~6.5 with and without the TS analog) and other observables such as the pK_a of Asp 102 in the absence of the TS analog (pK_a = 3 compared to observed pK_a of ~2). This finding establishes that the ΔpK_a values in the TFK system are inconsistent with the LBHB proposal (see also Discussion).

It is important to note that it is simply impossible to perform the analysis of Figures 6 and 7 by any current experimental approach. On the other hand, the analysis of Figures 6 and 7 is consistent with the available experimental information and involves reliable and well-tested electrostatic calculations. In our view, the ability to reproduce

TABLE I. Calculated and Observed pK_a Values of the Catalytic Groups in Chymotrypsin for the Free Enzyme and the Chymotrypsin/TFK Complex[†]

Case calculated	$pK_a^{(calc,A)a}$	$pK_a^{(calc,B)a}$	$pK_a^{(calc,C)a}$	$pK_a^{(calc)b}$	pK_a^{obs}
$[AHImH^+t^- \rightarrow A^-ImH^+t^-]$	−1.6	−0.8	−1.5	−2.2	
$[A^-ImHt^- \rightarrow A^-ImH^+t^-]$	10.3	10.6	10.8	12.9	12.0[19]
$[AHImt^- \rightarrow AHImH^+t^-]$	3.7	2.3	2.1	3.4	
$[AHImt^- \rightarrow A^-Imt^-]$	5.5	6.9	7.3	7.1	
$[AHImH^+ \rightarrow A^-ImH^+]$	−2.1	−0.2	−1.6	0.7	1.4[62]
$[AHIm \rightarrow A^-ImH^+]$	7.7	7.0	7.4	7.0	7.3[62]
$[AHIm \rightarrow AHImH^+]$	2.3	2.7	2.6	3.5	
$[AHIm \rightarrow A^-Im]$	4.7	4.6	3.9	2.8	

[†]Im, A, and t^- designate, respectively, His57, Asp102, and the covalently bound TFK inhibitor.
[a]Set A, B, and C are the different sets described in the PDLD/S-LRA Method section. The calculated pK_a values are apparent pK_a values evaluated with $\varepsilon_p = 4$ and the ε_{eff} of Ref. 32.
[b]These pK_a values are derived from the solvation calculations (taken from set C in Table II) and were transformed into pK_a values by the formula $\Delta G_i^p = -2.3RT\bar{q}_i(pK_{app,i}^p - pH)$ with pH = 5.

TABLE II. Calculated Free Energies of Different Configuration of the Catalytic Groups in Chymotrypsin for the Free Enzyme and the Chymotrypsin/TFK Complex[†]

Case calculated	$\Delta G^{(calc,A)\,a}$	$\Delta G^{(calc,B)\,a}$	$\Delta G^{(calc,C)\,a}$	$\Delta G^{(calc)\,b}$	ΔG^{obs}
$[AHImH^+t^- \rightarrow A^-ImH^+t^-]$	−7.7	−10.1	−8.6	−8.1	−9.0[16]
$[A^-Imt^- \rightarrow A^-ImH^+t^-]$	−10.4	−11	−9.3	−7.8	−9.7[19] [c]
$[AHImt^- \rightarrow A^-ImH^+t^-]$	−7.5	−6.9	−7.6		
$[AHImt^- \rightarrow AHImH^+t^-]$	2.6	2.2	2.1	3.7	
$[AHImt^- \rightarrow A^-Imt^-]$	2.5	2.9	3.1	2.6	
$[AHImH^+ \rightarrow A^-ImH^+]$	−6.5	−6.0	−5.5	−7.2	−5.0[62] [c]
$[A^-Im \rightarrow A^-ImH^+]$	−4.9	−2.8	−3.4	−2.8	−3.2[62] [c]
$[AHIm \rightarrow A^-ImH^+]$	−5.6	−5.2	−5.0		
$[AHIm \rightarrow AHImH^+]$	1.9	2.1	2.6	5.0	
$[AHIm \rightarrow A^-Im]$	−3.3	−2.7	−2.6	−6.0	

[†]Im, A, and t^- designate, respectively, His57, Asp102, and the covalently bound TFK inhibitor. The reported values were obtained by evaluating the changes of "soluation" free energies of the indicated configurations.
[a]Set A, B, and C are different sets described in the PDLD/S-LRA Method section.
[b]Obtained from the calculated pK_a values using $\Delta G_i^p = -2.3RT\bar{q}_i(pK_{app,i}^p - pH)$ for pH = 5.
[c]Obtained from the observed pK_a values using $\Delta G_i^p = -2.3RT\bar{q}_i(pK_{app,i}^p - pH)$ for pH = 5.

the observed pK_a values using a realistic model of the protein-inhibitor system is the best indication for the validity of our analysis.

EVB Calculations of the Free Energy Profile for PT Between His 57 and Asp 102

In principle, the simplest way to examine the LBHB hypothesis is to evaluate the free energy profile for PT between the given donor and acceptor. Such calculations are particularly effective as a complement to the PDLD/S-LRA calculations. Our EVB calculations of the PT between His57 and Asp102 are summarized in Figures 8 and 9. As seen from the figures, the calculation for both the free enzyme and the TFK system reproduces the trend obtained in our early studies.[13,16] In both cases, the PT is an uphill process, and the corresponding free energy surface does not correspond to an LBHB system. In particular, it is significant that the PT in the TFK is more exothermic than in the free enzyme. This finding shows that the LBHB character is smaller in the TS than in the free enzyme.

The PT free energy profile in serine proteases has been explored by ab initio QM/MM calculations[38,39] and by calculation of subsystems embedded in a continuum

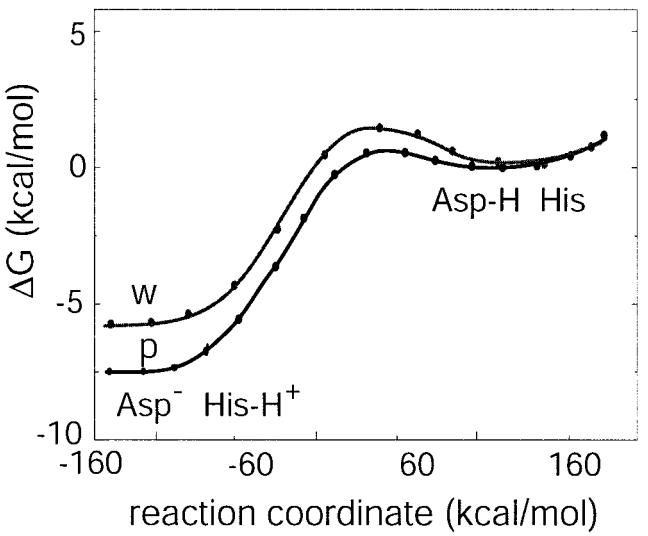

Fig. 8. EVB/US free-energy profiles for the proton transfer between a protonated His57 and an ionized Asp102 in the free enzyme (p) and in water (w). The reaction coordinate is taken as the energy gap between the equivalents of the fifth and third diabatic states of Figure 5 with neutral Ser195 instead of the TSA.

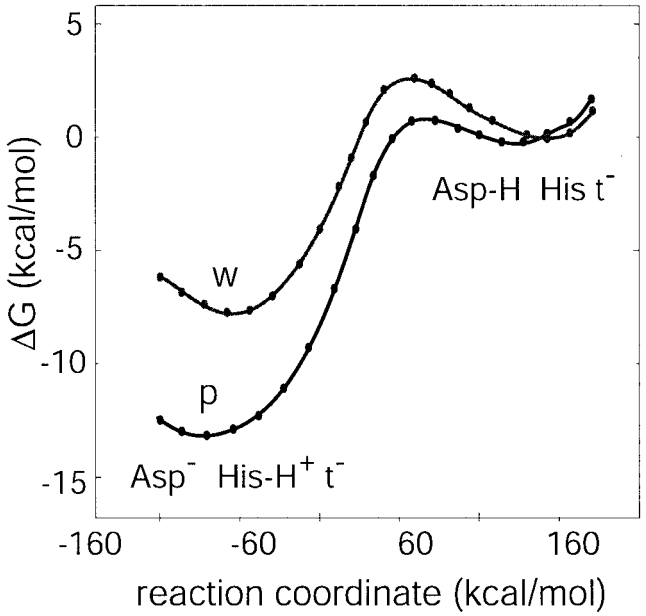

Fig. 9. EVB/US free energy profiles for the proton transfer between the protonated His57 and the ionized Asp102 in the enzyme with the bound TFK (p) and in water (w). The reaction coordinate is taken as the energy gap between the fifth and third diabatic states of Figure 5.

model.[40] These studies are consistent with our early EVB studies (e.g., see Ref. 13) and with the present study. However, the present study is more relevant to the test case of the chymotrypsin/TFK system. Furthermore, as much as the QM/MM studies are concerned, we believe that these studies do not yet reflect sufficient configurational averaging (see discussion in Ref. 41). We also consider a calibration (or validation) on the reference water reaction as an essential part of the analysis of the PT energetics, and such a calibration has not been reported in the available QM/MM studies. Thus, the fact that our EVB study was calibrated on the experimental energies of the PT reaction in water (Figs. 8 and 9) is one of the main reasons for our trust in the conclusions of this study.

DISCUSSION

The results obtained in the previous section provide a clear illustration of the inconsistency of the LBHB proposal. The general significance of these findings and their relationship to other aspects of the LBHB proposal is discussed below.

The LBHB Proposal Is Inconsistent With the Energetics of the TFK System

This work explored the validity of the LBHB hypothesis in serine proteases using the TFK system as a benchmark. After defining the LBHB proposal in a way that distinguishes it from the previous proposal of preorganized ionic HBs,[5] we pointed out that all experimental observations used to support the LBHB idea are at least equally consistent with the ionic HB model. We also explain why asymmetric HBs are inconsistent with the LBHB model.

TABLE III. Calculated Changes in Solvation Energies (in kcal/mol) for Typical Changes in the Ionization States of the Free and TFK-Bound Enzyme†

Case calculated	ΔG^{w}_{sol}	ΔG^{p}_{sol}
[AHImH → A^-ImH]	−80	−85
[AHIm → $AHImH^+$]	−65	−55
[$AHImH^+t^-$ → $A^-ImH^+t^-$]	−73	−108
[A^-Imt^- → $A^-ImH^+t^-$]	−49	−72

†Im, A, and t^- designate, respectively, His57, Asp102, and the covalently bound TFK inhibitor. The ΔG_{sol} values correspond to the processes described in the corresponding "case calculated" column.

With this in mind, we clarify that the best way to resolve the ionic HB versus the LBHB proposal is to consider the relevant energies and pK_a values (an issue that is mostly ignored by the LBHB proponents). Both the PDLD/S-LRA and EVB calculations gave a positive free energy for the PT from His57 to Asp102 with and without the TS analog. Thus, the pK_a shift is quantitatively consistent with a model where there is a very small covalent character in the free enzyme and an even smaller character in the enzyme with the TS analog. In addition, it is clear from the enormous contribution of the calculated solvation energy (Table III and Ref. 16) that the environment here is very polar and does not resemble the nonpolar environment envisioned by the LBHB proponents. The fact that the PDLD/S-LRA calculations reproduce the observed pK_a values in the system studied provides a significant credibility to the rest of the analysis of Figures 6 and 7, which could not be performed by direct pK_a measurement.

As is clear from the above analysis, we do not have a symmetric LBHB in both the water and the protein systems. Yet it is instructive to examine the covalent mixing of the system. This can be done by using Eqs. 4 and 5, where in the present case Φ_1 = [O^- H-N^+] and Φ_2 = [O-H N], but all the considerations of Defining the Problem section still hold. Now using the EVB calculations and Eq. 5 we obtain C_2^2 values of 0.1, 0.16, and 0.16 for the reaction in water, free enzyme, and the enzyme/TFK system. As seen from this result, and as pointed out by one of us long ago,[14,24] all systems studied (including the reaction in water) have covalent character. Although both the reaction in water and in the enzyme have covalent character, it is unclear (see below) whether the covalent character contributes to catalysis, which is the difference between the reaction in the enzyme and in water.

At this stage, it is important to realize a point, which is frequently ignored in discussing the LBHB proposal. That is, even if the partial covalent character in the enzyme is somewhat larger than in solution, we must examine whether this contributes to catalysis. Here we have to compare the free energy of moving from the ground to the TS in the enzyme and in solution with and without the covalent mixing. This comparison can be simplified (and approximated) by examining the effect of the H_{ij} that mixes states 3 and 5 of Figure 5 in the PT process (it is assumed that the LBHB effect on state 1 is small). Here it was found that the adiabatic and diabatic values of $\Delta\Delta G_{PT}^{w\rightarrow p}$ are similar for the TFK system. Thus, the in-

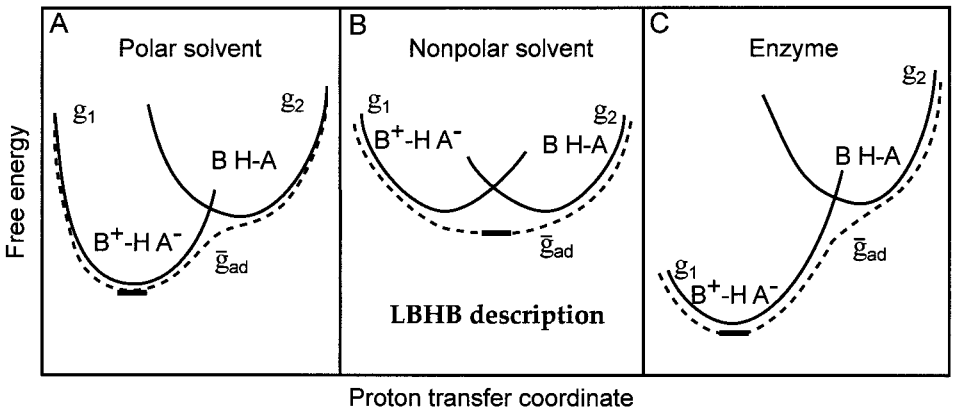

Fig. 10. Schematic representation of the free energy surface perpendicular to the actual reaction coordinate at the transition state of the hydrolysis reaction catalyzed by serine protease. The proton transfer (PT) coordinate corresponds to the position of the proton between Asp^- and His^+ of the "catalytic triad." The lowest point (the dark line) on each adiabatic free energy curve ($\bar{g}_{ad}$) corresponds to the transition state of the catalytic reaction [ts(a) or ts(b) in Fig. 1] of the given system. Note that the relevant reaction is not along the PT coordinate (see Ref. 14 for more details).

crease in $\Delta\Delta G_{PT}^{w\rightarrow p}$ and the corresponding catalytic contribution is due to electrostatic rather than to a covalent mixing effect.

The conclusions of the present findings about the LBHB proposal in serine protease are in agreement with our previous considerations[13,14,16] and can be summarized by the schematic description of Figure 10. This figure (see also Ref. 14) illustrates the fact that when the TS of an enzyme involves a system that forms an ionic HB in solution (e.g., B^+HA^-), it will always make the ionic system more stable (relative to solution) because this will lead to a larger TS stabilization. Increasing the stabilization of the ionic state will decrease rather than increase the LBHB character of the system. It is encouraging to note that this point is strongly supported by the present calculations.

The calculations of the profile for the PT between His57 and Asp102 (Figs. 8 and 9) are consistent with our previous findings (Fig. 5 of Ref. 13) and with recent QM/MM studies.[38,39] We point out, however, that despite the useful implementation of ab initio QM/MM calculations in Refs. 38 and 39 and despite the similarity between the conclusions of Refs. 38 and 39 and our study, we consider the EVB study to be more conclusive. That is, obtaining quantitative results by ab initio QM/MM studies requires major configurational sampling,[41,42]which has not yet been accomplished in studies of serine proteases. Here it is crucial to show that the calculations reproduce both the experimental information about the corresponding solution process and the observable pK_a values in the protein. Doing so is perhaps the key accomplishment of the present work. It is also useful to clarify that all quantum mechanical calculations that supported the LBHB hypothesis involved incorrect treatments. This ranges from confusing gas phase calculations with studies of enzymes[27,43,44] to more sophisticated studies that involved, however, very limited subsystems (e.g., see Ref. 45) (for more discussion, see Refs. 3 and 46). At any rate, it seems clear to us that the LBHB proposal must be explored by calculations that consider the entire protein solvent system and its proper configurational averages. Our experience with EVB studies show that such ab initio calculations will show that the LBHB effect is not a major catalytic factor.

Although the present work focuses on the LBHB proposal in serine proteases, it is important to consider the validity of this proposal in other systems. Apparently, the LBHB proposal is not supported by calculations that properly considered the effect of the protein environment. This includes EVB studies of the keto-enol isomerization reactions[47] and the oxyanion hole in subtilisin[14] as well as QM/MM studies of mandelate racemase[48] and citrate synthase.[49]

Experimentally Based Definitions of LBHBs Are Consistent With Ionic HBs

The LBHB proponents have brought repeatedly experimental observations as *definitions* of the LBHB proposal (e.g., see Refs. 12 and 26). This involves significant problems and circular arguments (e.g., see Ref. 50), because the LBHB model can only be established by the *interpretation* of the experiment, rather than by the experimental observations themselves. In this respect, it is important to emphasize that all the experimental evidences brought so far in support of the LBHB hypothesis are equally consistent with the idea of an ionic HB (see also analysis in Refs. 14 and 50). A case in point is the unusual low-field signal that is seen for the proton between His57 and Asp102 at low pH, as well as in transition-state analogs of chymotrypsin, trypsin, subtilisin, and α-lytic protease.[19,20,51] Although these low-field shifts were brought as evidence of the LBHB proposal, it is clear that we are dealing here with an arbitrary interpretation of experimental observations. That is, as stated in Ref. 14, the low-field shift can be due to the electrostatic effect of $Asp102^-$ on $His57^+$. This point has been in fact established by recent QM/MM calculations[38] and in calculations of a model system.[52] Furthermore, NMR studies[17,18] have shown clearly that

the proton is at least 85% localized on $N^{\delta 1}$ rather than delocalized. Furthermore, the LBHB proposal implied that the donor and acceptor are in a nonaqueous hydrophobic environment with a very small pK_a difference. Now the NMR experiments of Bachovchin and coworkers[17,18] have indicated that the environment is not hydrophobic, and (more significantly in our view) any reasonable calculation shows that the environment around the Asp His pair is polar (see below).

Significant experimental effort has been invested in the determination of the bond length between $N^{\delta 1}$ of His57 and the hydrogen that forms the HB with Asp102. The pioneering neutron diffraction study of Kossiakoff and Spencer[53] concluded that the hydrogen is bonded to the histidine. A recent high-resolution study of Kuhn and coworkers[54] concluded that the N-H bond length is 1.2 Å (the regular bond length is 1.08 Å) and that the O-H bond length is 1.5 Å. An NMR study of Bachovchin and coworkers[18] concluded that the N-H distance is ~1.113 Å in the native enzyme at low pH and 1.085 Å in a TSA complex. Thus, *all* structural studies are consistent with a much larger contribution of the ionic HB than the LBHB (note the above comment about the inconsistency of the idea that an LBHB can be asymmetric). It is also important to note that the X-ray study did not involve a TSA, which is expected to have an even smaller LBHB as established in this work. The finding of a short N–O distance (2.6 Å) is completely consistent with ionic HBs (see above).

Another misunderstanding is associated with the attempt to relate the LBHB proposal to the observation of a small H/D fractionation factor (e.g., see Ref. 26). That is, it is frequently stated that LBHBs are associated with a small fractionation factor, whereas other asymmetric HBs presumably lead to large fractionation factors. This interpretation is based on an arbitrary assumption[55] about the corresponding shape and curvature of the HB potential surfaces (and thus on the corresponding zero point energy). Unfortunately, no attempt was made by the LBHB proponents to examine the potential surfaces of ionic HBs (with a shallow minimum) and the corresponding zero point energy, which would lead to a small fractionation factor. In fact, this point has been established by a recent theoretical analysis.[56] Another closely related example of the unjustified assumption, brought to support the LBHB proposal, is the assertion that the NMR H/D and H/T isotope shifts can be used for unique assignments of LBHBs. Here it is customary to assume that the isotope shift reflects the average position of the H, D, or T isotopes and that this can be used to deduce the existence of an LBHB-type of potential surface (e.g., see Ref. 57). What is missing from this argument is a demonstration that the potential surface of an asymmetric ionic HB does not lead to the same NMR isotope shift. In other words, unless one calculates the isotope shifts for the LBHB and ionic HB surfaces, they cannot provide any unique interpretation of the NMR observations.

It is instructive to consider the nature of ionic HBs in solutions as a further clarification of the LBHB proposal. The impressive experimental studies of Refs. 58 and 59 have provided insightful information about the difference between ionic and neutral HBs in different solvents[58] and about the difference between tight and "open" ionic HBs.[59] However, this did not provide direct information about LBHB in solution. That is, the equilibrium between ionic and neutral HBs (e.g., AH $BH \rightleftharpoons [AH\ B]^- + H^+$) does not tell us what the delocalized and localized contributions are to the ionic HB (e.g., what is the relative contribution of the localized $[A^-HB]$ and the delocalized $[A^{-\delta} \cdots H \cdots B^{-\delta}]$ forms).

It is also useful to comment about the existence of LBHBs in different solvents. As clarified in Defining the Problem section, the LBHB proposal implies a fully or almost fully delocalized system with $\Delta G_{PT} \simeq 0$ and with a symmetric potential. However, the careful experimental studies of Perrin and Ohta[60] did not find any symmetric HB while examining the LBHB hypothesis in different solvents.

CONCLUSIONS

The LBHB proposal is frequently presented as a controversial proposal with supposedly equally valid arguments on both sides (e.g., see Ref. 2). This perception is largely due to the tendency to accept "soft" definitions as valid scientific arguments and to the tradition (which was justified in the early days of enzymology) to present catalytic proposals without resorting to unique definitions (see discussion in Ref. 61). The present work addresses the LBHB proposal while insisting on the use of a unique verifiable definition for this proposal and the alternative proposal of ionic HBs. With the VB-based definition, we show that the description of the LBHB proposal in terms of strength and distance does not provide a valid definition. We also clarify that all the experimentally based definitions used by the LBHB proponents are equally consistent with ionic HBs. Furthermore, it is pointed out that the LBHB proposal is inconsistent with the finding of asymmetric HBs. It is then clarified that only energy-based considerations can be used in a unique way in resolving the LBHB controversy, because this is an issue of energetics of competing resonance structures and the relative contribution of these resonance structures to catalysis. Thus, the determination of the relevant ΔG_{PT} (or ΔpK_as values) is the clearest (and perhaps the only) way of examining the LBHB proposal.

With the above background in mind, we examine the energetics of one of the most important test cases provided by the LBHB proponents, namely, the TFK system. It is found that the TFK system, which is an excellent model of the TS of serine proteases, corresponds to an ionic HB rather than to an LBHB. That is, we demonstrate that the ΔpK_a between the donor and acceptor, which defines the proposed LBHB (Asp102 and His57), increases rather than decreases on binding of the TSA. Establishing this point by well defined energy considerations provides a clear demonstration of the power of energy-based analysis.

The present analysis might be considered by some as a biased analysis, because it did not use experimental criteria for excluding the LBHB proposal. However, we

used here what we consider as the only valid way of resolving the LBHB argument. That is, we first reproduced the observed pK_a values (this work) and mutational effects (Ref. 13) and then used the same theoretical model to examine the proton transfer energy between the donor and acceptor of the HB in question. We hope that our study will inspire the LBHB proponents to use verifiable definitions and to address the issue of ΔpK_a in what they consider to be LBHB candidates.

ACKNOWLEDGMENTS

We thank Dr. Elena Cubero i Jordà for her contributions in the initial step of this project.

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