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Theoretical study of the role of low-barrier hydrogen bonds in enzyme catalysis: a model of proton transfer in serine protease

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Abstract. A model of low-barrier hydrogen bonds (LBHBs) in enzymes has been studied by ab initio quantum mechanical calculations including the self-consistent reaction field solvent model. The hydrogen-bond strengths and the deprotonation energies for the hydrogen-bonded and non-hydrogen-bonded *cis*-urocanic acid were calculated at the HF/6-31 + G(d,p) level at various dielectric constants. The same calculations were performed for the α,β-dihydrourocanic acid to model the catalytic dyad of serine protease. The deprotonation energy of $N^{\varepsilon 2}$ in α,β-dihydrourocanic acid is increased by formation of LBHBs and depends very much on the dielectric constant. This study suggests that the formation of LBHBs increases the basicity of the dyad, and the polarity change near the reaction center in the active site could help the proton abstraction from Ser 195 and the donation to the leaving group. Both the LBHBs and the environment can play crucial roles in the enzyme catalysis.

Key words: Low-barrier hydrogen bond – Enzyme catalysis – Serine protease – Catalytic dyad – Environmental effect

1 Introduction

Proton transfer is an important phenomenon in chemistry and biology. Proton transfer, in general, has a high-energy barrier, but for a number of hydrogen-bonded systems it has only a very low barrier, leading to a proton delocalization in the hydrogen bond that strongly depends on the environments. Such hydrogen bonds are called "short strong" or "low-barrier" hydrogen bonds (SSHBs or LBHBs), and it has recently been proposed that they may provide an unusually large amount of stabilization to high-energy enzyme-bound intermediates and/or transition states [1, 2]. There has been considerable debate recently about the existence, the strength, and the role of LBHBs in enzymes. There are some workers who believe that enzyme activity can be understood by considering mainly electrostatic pair potentials [3, 4, 5]; however, we do not believe this view is valid, and recent results seem to support the existence and importance of SSHBs in enzymes [6, 7]. In addition, recent NMR studies have shown that hydrogen bonds in proteins have covalent character, especially when the proton affinities of the two bases are similar [8, 9]. We believe that the present results provide more evidence for the validity and utility of the SSHB concept.

The hydrogen bond between the Asp and His residues in the catalytic triad of serine protease has been presented as an example of the potential contribution of LBHBs to enzyme catalysis [10, 11, 12, 13, 14, 15, 16, 17, 18, 19]. A schematic diagram of the catalytic mechanism of serine protease is shown in Fig. 1. Frey and co-workers [10, 11, 12] have suggested that the LBHB in the protonated dyad (Asp–His) should increase the basicity of His 57 strongly enough to abstract a proton from Ser 195. The basicity of His 57 should be decreased again later to give a proton from its conjugate acid form to a leaving group. They have reported that the pK_as of Ser 195 and the leaving amino group in chymotrypsin are about 14 and 9, respectively, and that the experimental pK_as of the protonated dyad in the active site bound to two different trifluoromethyl ketones, *N*-acetyl-L-Leu-DL-Phe-CF_3 (*N*-AcLF-CF_3) and *N*-acetyl-DL-Phe-CF_3 (*N*-AcF-CF_3), are 12.0 and 10.8, respectively [11, 18]. Since these values are between 14 and 9, it was suggested that the dyad could abstract a proton from Ser 195 as a general base and donate one to the leaving amino group as a general acid [11]. They have also suggested that the binding of a substrate induces a conformational change in the enzyme that leads to steric compression between His 57-$N^{\delta 1}$ and Asp 102-O^{γ}. This compression would be relieved by LBHB formation, which is possible only when His 57 is protonated on $N^{\varepsilon 2}$. Therefore the strain of compression between His 57 and

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Asp 102 increases the basicity of His 57. In this study we propose another possible mechanism of action in the enzyme active site associated with the role of LBHBs.

Enzyme catalysis depends very much on the environment of the active site, which provides not only an appropriate dielectric medium but also many specific interactions for the catalysis. It has long been a goal of many scientists to understand the role of the active site; however, it is not completely understood yet. The dielectric property of the active site is not homogeneous and depends much on the orientation of functional groups, and it may even be changed during the enzymatic reaction. The strength of a LBHB depends very much on the dielectric medium [20, 21], so the role of the LBHB may be changed accordingly during the course of the reaction. In this study we focus on the relative basicity of the catalytic dyad depending on the formation of a strong hydrogen bond. The schematic potential-energy curves for proton abstraction from Ser to the dyad with a SSHB and without are shown in Fig. 2. The relative energetics between the potential curves for Fig. 2a and b are independent of the energies of Ser–OH and Ser–O^-, since their energies are canceled out. Therefore the deprotonation energies for the protonated dyads (strongly hydrogen bonded and not hydrogen bonded) will give a good estimate for the relative basicity. The basicity of the dyad will depend on the environment, so it is necessary to study the role of the local polarity on the relative basicity. Since we are interested in the relative energetics in term of the formation of a SSHB as illustrated in Fig. 2, there is no problem in the state of the reference in this study.

A *cis*-urocanic acid was used as a model of the dyad [10, 22], and it is known to form a strong hydrogen bond. The intramolecular hydrogen bond in this model is slightly different from that in serine protease: the carboxy group is an anti form in the model, while it is a syn form in the enzyme. The hydrogen bond strength of the *cis*-urocanic acid was estimated to be about 5 kcal-mol^{-1} in the acetone–water cosolvent [22]. The LBHB forms a charged species in which the charge may be delocalized over several atoms; thus, some component of the interaction will be electrostatic and, therefore, the strength of the interaction will depend on the local dielectric constant. In order to study the role of the LBHB in the active site, where its local dielectric constant is changed along the reaction pathway, we performed ab initio quantum mechanical calculations for the *cis*-urocanic acid including a solvent effect with various dielectric constants. We used a thermodynamic cycle in this study as shown in Fig. 3, and this cycle was

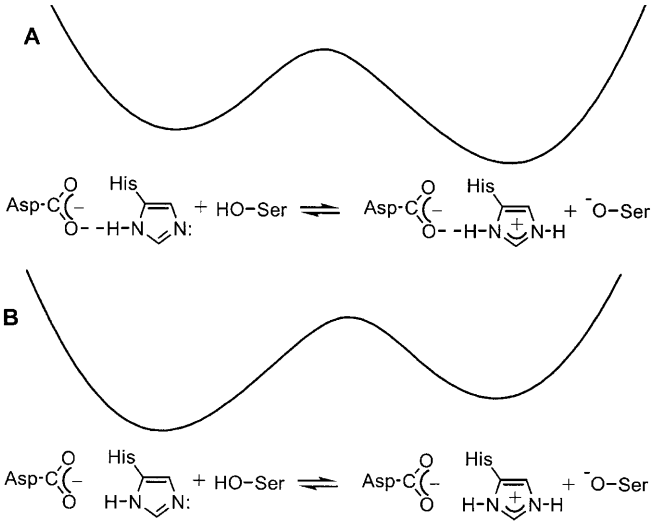

Fig. 2. Schematic potential-energy curves for the proton abstraction from Ser 195 to **(A)** the strongly hydrogen-bonded dyad and **(B)** the non-hydrogen-bonded dyad in a given environment. The relative energetics between **A** and **B** depends on the basicity of His

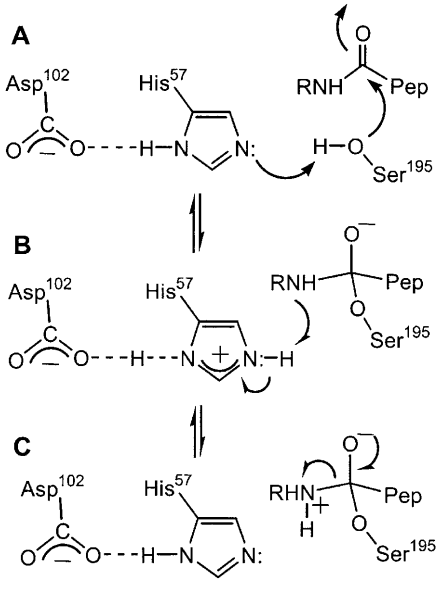

Fig. 1. Schematic diagram for the mechanism of proton transfer in serine protease

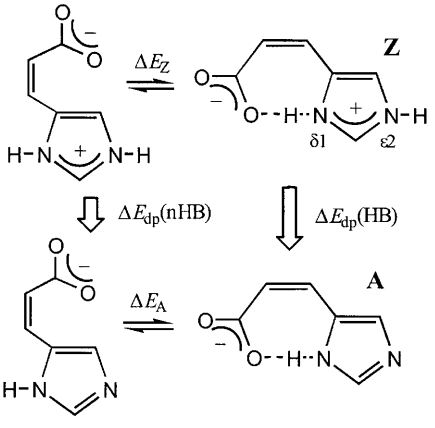

Fig. 3. Schematic diagram for the deprotonation and the formation of low-barrier hydrogen bonds in the *cis*-urocanic acid. ΔE_Z and ΔE_A are the relative energies for the hydrogen-bonded zwitterionic (Z) and the anionic (A) forms of the *cis*-urocanic acid with respect to the corresponding non-hydrogen-bonded structures, respectively. They are the hydrogen-bond strengths for the Z and A forms of the *cis*-urocanic acid. ΔE_{dp}(nHB) and ΔE_{dp}(HB) are deprotonation energies of $N^{\varepsilon 2}$ protons for the non-hydrogen-bonded and hydrogen-bonded *cis*-urocanic acids, respectively

used to model the enzymatic process depicted in Fig. 2. The strengths of the LBHBs were calculated and compared with experimental values. The hydrogen bond strengths of the α,β-dihydrourocanic acid, which mimics the dyad better, were also calculated with various dielectric constants, and these values are used to model the role of the LBHB in an enzyme.

Computational methods

All the electronic structure calculations were done using the GAUSSIAN 94 quantum mechanical package [23]. The geometries for the ***cis***-urocanic acid were optimized at the Hartree–Fock (HF) level of theory using the 6-31 + G(d,p) basis set in the gas phase. The geometries in a dielectric medium were optimized at the HF/6-31 + G(d,p) level using the Onsager dielectric continuum model [24], and they were used to obtain energies from single-point calculations using the polarized continuum model [25], namely the isodensity polarized continuum model (IPCM) and the self-consistent IPCM (SCIPCM) with the isodensity value of 0.0004. In the reaction field theory, the solute in a cavity is surrounded by a polarizable medium with a dielectric constant. A dipole in the solute induces a dipole in the medium, and the electric field applied to the solute by the solvent dipole interacts with the solute dipole to produce net stabilization. The cavity radius is an adjustable parameter, and the choice of the radius has been discussed extensively [26, 27, 28, 29]. In the Onsager model, the radius was calculated from the molecular volume of the optimized structure in the gas phase, on the assumption that the structure is spherical, and was increased by 0.5 to consider the surrounding solvent molecules. In the IPCM, the cavity is defined as an isosurface of the electron density. The isodensity surface is determined by an iterative process in which a self-consistent-field (SCF) cycle is performed and converged using the current isodensity cavity. The resulting wavefunction is used to update the isodensity cavity, and this procedure is repeated until the cavity shape changes no longer upon completion of the SCF. However, the terms that couple the isodensity to the solute Hamiltonian are missing in this process. In the SCIPCM, the SCF procedure solves the electron density which minimizes the energy, including the solvation energy that depends on the cavity, which depends on the electron density again. Therefore, the effects of solvation are folded into the iterative SCF calculation. The SCIPCM thus accounts for the full coupling between the cavity and the electron density and includes terms that the IPCM neglects.

Results and discussion

The cis-urocanic acid

The geometries and energies for the hydrogen-bonded and non-hydrogen-bonded conformers as depicted in Fig. 3 were calculated at the HF/6-31 + G(d,p) level of theory both in the gas phase and in solution using the Onsager SCRF method. The optimized geometries are all planar. The bond lengths for O–H and N–H, which are denoted as r_Z(O–H) and r_Z(N–H) for the zwitterionic form (Z form) and r_A(O–H) and r_A(N–H) for the anionic form (A form), are listed in Table 1. It was not possible to obtain the hydrogen-bonded structure of the Z form in the gas phase and at $\varepsilon = 2.0$, since the neutral form in which the proton is transferred to the carboxyl group is the most stable structure. The bond lengths for O–H and N–H in terms of the Onsager function, $(\varepsilon-1)/(2\varepsilon + 1)$, are plotted in Fig. 4. All the bond lengths are linear in this plot. If we extrapolate the r_Z(O–H) and r_Z(N–H) values to $\varepsilon = 2.0$, we can obtain about 1.20 and 1.12 Å for them, respectively; however, they are very short, so a proton jumps to the carboxyl groups before they come to such short distances. The hydrogen-bond length for the Z form, r_Z(O–H), was 1.50 Å at $\varepsilon = 5.0$, which is very short and comparable to those of LBHBs reported in the literature [1, 30, 31, 32]. The N–H–O distance at $\varepsilon = 5.0$ is 2.51 Å, which agrees very well with that in the *N*-AcLF bound enzyme complex [33]. This distance increases with the dielectric constant. The hydrogen-bond length of O–H for the A form was 1.68 Å in the gas phase, and this distance also increases with the dielectric constant as shown in Fig. 4. The hydrogen-bond lengths in the Z form, denoted by r_Z(O–H), depend more on the dielectric constant than those in the A form. The r_Z(O–H) value is smaller and the r_Z(N–H) value is larger than the corresponding values in the A form at the same dielectric constant. This means that the proton in the Z form is delocalized more than that in the A form, which is consistent with the NMR experiments. The large downfield chemical shift of the $N^{\delta 1}$ proton in the *cis*-urocanic acid has been observed when the Z form of the *cis*-urocanic acid is formed from the A form, which was attributed to the formation of stronger and more delocalized LBHBs [22].

Table 1. Bond lengths for low-barrier hydrogen bonds (*LBHBs*) in the Z and A forms of the *cis*-urocanic acid optimized at the HF/6-31 + G(d,p) level using the Onsager dielectric continuum model. Lengths are in angstroms

ε[a]	r_Z(N–H)	r_Z(O–H)	r_A(N–H)	r_A(O–H)
2	b	b	1.026	1.726
5	1.064	1.498	1.011	1.771
10	1.037	1.621	1.010	1.789
20	1.028	1.678	1.008	1.808
78.4	1.023	1.723	1.008	1.818

[a] Dielectric constant
[b] It was not possible to optimize the geometry

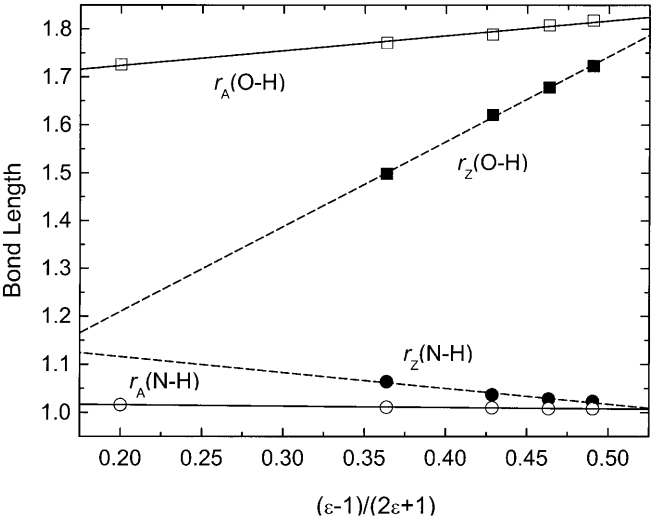

Fig. 4. Calculated bond lengths for the hydrogen-bonded Z and A forms of the *cis*-urocanic acid in terms of the Onsager function

The strengths of the LBHBs were obtained from the energy differences between the hydrogen-bonded and the non-hydrogen-bonded conformers of the *cis*-urocanic acid as shown in Fig. 3. They are denoted by ΔE_Z and ΔE_A for the Z and A forms, respectively, and are listed in Table 2. The ΔE_Z and ΔE_A values calculated at different dielectric constant are plotted in Fig. 5. The ΔE_Z and ΔE_A values at $\varepsilon = 20.0$ from the IPCM were −4.33 and −4.64 kcal mol^{-1}, respectively. They agree very well with the experimental value [22]. The calculated hydrogen-bond strengths for the Z and A forms using the IPCM are approximately the same. The ΔE_Z and ΔE_A values at $\varepsilon = 20.0$ from the SCIPCM were −9.43 and −7.75 kcal mol^{-1}, respectively, which are smaller than those from the IPCM. The difference between the ΔE_Z and ΔE_A values is about 1.68 kcalmol^{-1}, which is not very large, but agrees well with the experimental value [22]. The SCIPCM slightly overestimates the hydrogen-bond strengths of the *cis*-urocanic acid, but reproduces the difference in the hydrogen-bond strengths between the Z and A forms better.

One of the proposed roles of the LBHB is to increase the basicity of the dyad to abstract a proton from Ser 195.

Table 2. The formation energies of LBHBs in the dielectric medium at the HF/6-31+G(d,p) level using the isodensity polarized continuum model (*IPCM*) and the self-consistent IPCM (*SCIPCM*). The energies were calculated from the structures optimized using the Onsager dielectric continuum model. The *numbers* in *parentheses* are from the SCIPCM

ε[a]	ΔE_Z (kcal mol^{-1})	ΔE_A (kcal mol^{-1})
Gas	–	−16.96
2	−13.10 (−15.22)[b]	−12.06 (−13.43)
5	−7.04 (−11.14)	−7.70 (−10.07)
10	−5.20 (−9.85)	−5.76 (−8.59)
20	−4.33 (−9.43)	−4.64 (−7.75)
78.4	−3.82 (−9.37)	−3.72 (−7.08)

[a] Dielectric constant
[b] The energies of the hydrogen-bonded Z form were obtained by using the geometry optimized at ε = 5.0

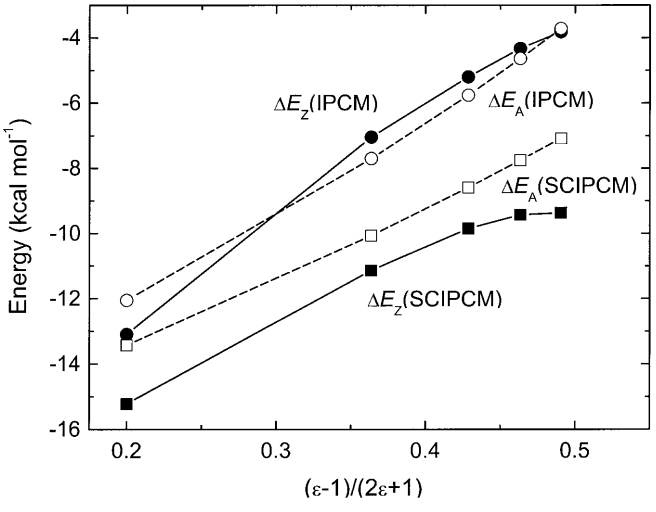

Fig. 5. The hydrogen-bond strength for the Z and A forms of the *cis*-urocanic acid in terms of dielectric constants

In order to understand how it happens, we calculated the deprotonation energies for the $N^{\varepsilon 2}$ protons of the hydrogen-bonded and non-hydrogen-bonded *cis*-urocanic acids in a dielectric medium. The deprotonation energies for the hydrogen-bonded and non-hydrogen-bonded acids, denoted as ΔE_{dp}(HB) and ΔE_{dp}(nHB), respectively, are obtained from the relative energies of the Z forms of the *cis*-urocanic acid with respect to the A forms, and they are listed in Table 3. Garcia-Viloca et al. [34] have calculated the deprotonation energy for the conjugate acid of 1-methylimidazole at the HF/6-31+G(d,p)//6-31+G(d,p) level in the gas phase, which is 249 kcal mol^{-1}. This value is much smaller than the deprotonation energies for the *cis*-urocanic acid at $\varepsilon = 2.0$, which suggests that the basicity of the imidazole is largely increased. This result may originate from the formation of LBHBs, delocalization of the charge density, and the electrostatic stabilization of the Z form. The deprotonation energies are decreased when the dielectric constant is increased, and the differences between the ΔE_{dp}(HB) and ΔE_{dp}(nHB) values are not large. Ash et al. [22] have reported the pK_a difference between the *cis*- and *trans*-urocanic acids in acetone-water cosolvent. The ΔpK_a value was 0.85, which corresponds to about 1.2 kcal mol^{-1} in energy. The theoretical estimation for the difference in the deprotonation energy between the hydrogen-bonded and non-hydrogen-bonded *cis*-urocanic acids was 1.7 kcal mol^{-1} at $\varepsilon = 20$, which agrees well with the experimental value. This energy difference is identical to the difference in the hydrogen-bond strengths between the Z and A forms. These results suggest that the formation of LBHBs increases the basicity of the $N^{\varepsilon 2}$ proton by about 1.2 kcal mol^{-1}, experimentally, and 1.7 kcal mol^{-1}, theoretically.

The α,β-dihydrourocanic acid

The deprotonation energies and the hydrogen-bond strength are not very much different between the hydrogen-bonded and non-hydrogen-bonded *cis*-urocanic acids. This suggests that the delocalization of the charge density through the molecular backbone and the electrostatic stabilization seem to be more important to the change of pK_a than the formation of the LBHB. Because of these factors, the *cis*-urocanic acid may not be able to model the role of the LBHB in the catalytic dyad of serine protease correctly. So, we also performed the quantum

Table 3. Deprotonation energies for the hydrogen-bonded and non-hydrogen-bonded *cis*-urocanic acids in the dielectric medium at the HF/6-31+G(d,p) level with the SCIPCM. The energies were calculated from the structures optimized using the Onsager dielectric continuum model

ε[a]	ΔE_{dp}(HB)	ΔE_{dp}(nHB)
2	305.7[b]	304.0
5	301.0	299.9
10	299.8	298.6
20	299.4	297.7
78.4	299.1	296.8

[a] Dielectric constant
[b] The energies of the Z form were obtained by using the geometry optimized at ε = 5.0

mechanical calculations for the α,β-dihydrourocanic acid to avoid the delocalization of the charge density through the molecular backbone. The optimized geometries for the Z and A forms at the HF/6-31 + G(d,p) level of theory with the Onsager SCRF method are shown in Fig. 6. They are not planar, so there is no delocalization of the charge density through the molecular backbone. The lengths of the LBHBs in the Z and A forms of the α,β-dihydrourocanic acid are listed in Table 4 and they are plotted in terms of the Onsager function as depicted in Fig. 7. The O–H distances are longer than those of the *cis*-urocanic acid, which indicates that the hydrogen bonds become weaker by removing electron delocalization. For the A forms of acids at $\varepsilon = 5.0$, the O–H distance is increased by about 0.13 Å, from 1.77 to 1.90 Å, by removing delocalization, but for the Z forms it is increased by only about 0.04 Å. The hydrogen-bond length in the A form of the α,β-dihydrourocanic acid is within the range of the normal hydrogen bonds; however, the hydrogen-bond length in the Z form is still quite short, 1.54 Å at $\varepsilon = 5.0$, which agrees well with that of the LBHB in enzyme complexes [33].The difference in the hydrogen-bond strength between the Z and A forms is largely increased as shown in Table 5. The hydrogen bonds in the Z form at $\varepsilon = 2$, 5, and 10, are 11.8, 7.2, and 5.9 kcal mol^{-1} stronger, respectively, than those in the A form. These make the deprotonation energies for the hydrogen-bonded α,β-dihydrourocanic acid larger than those for the non-hydrogen-bonded form by the same amount as shown in Table 6. The LBHB at $\varepsilon = 5$ makes the

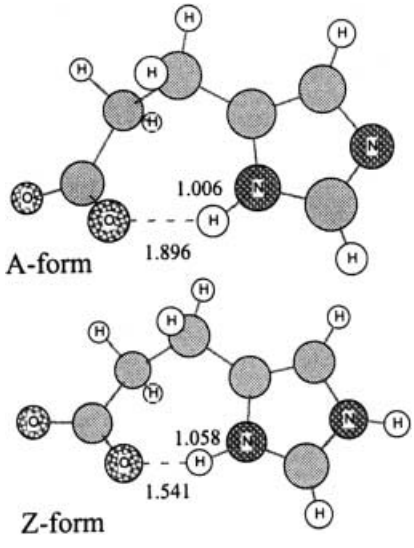

Fig. 6. The optimized structures for the A and Z forms of the α,β-dihydrourocanic acid using the self-consistent reaction field method with $\varepsilon = 5.0$ at the HF/6-31 + G(d,p) level of theory. The *numbers* are bond lengths in angstroms

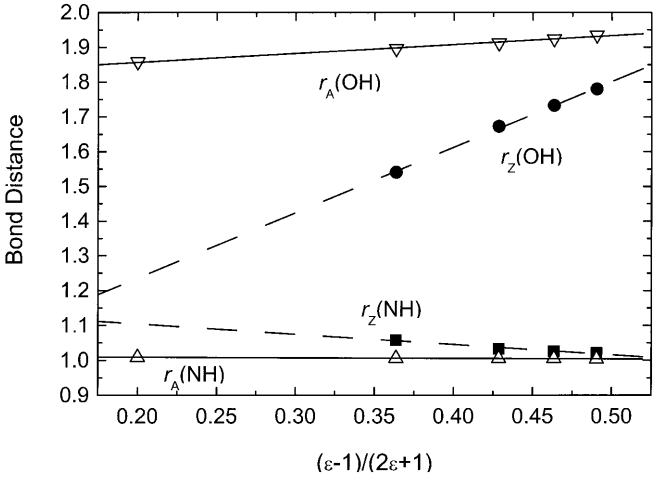

Fig. 7. Calculated bond lengths for the hydrogen-bonded - and A forms of the α,β-dihydrourocanic acid in terms of the Onsager function

Table 4. Bond lengths for LBHBs in the Z and A forms of the α,β-dihydrourocanic acid optimized at the HF/6-31 + G(d,p) level using the Onsager dielectric continuum model. The lengths are in angstroms

ε[a]	r_Z(N–H)	r_Z(O–H)	r_A(N–H)	r_A(O–H)
2	b	b	1.009	1.858
5	1.058	1.541	1.006	1.896
10	1.033	1.673	1.005	1.912
20	1.026	1.733	1.005	1.924
78.4	1.021	1.780	1.004	1.935

[a] Dielectric constant
[b] It was not possible to optimize the geometry

Table 5. The LBHB energies of the α,β-dihydrourocanic acid in the dielectric medium at the HF/6-31 + G(d,p) level using the IPCM and SCIPCM. The energies were calculated from the structures optimized using the Onsager dielectric continuum model. The *numbers* in *parentheses* are from the SCIPCM

ε[a]	ΔE_Z (kcal mol^{-1})	ΔE_A (kcal mol^{-1})
2	−18.50 (−18.96)[b]	−7.93 (−7.21)
5	−11.90 (−11.72)	−6.21 (−4.56)
10	−10.29 (−9.23)	−5.50 (−3.37)
20	−9.21 (−8.25)	−5.11 (−2.96)
78.4	−8.35 (−7.42)	−4.78 (−2.33)

[a] Dielectric constant
[b] The energies of the hydrogen-bonded Z form were obtained by using the geometry optimized at $\varepsilon = 5.0$

Table 6. Deprotonation energies for the hydrogen-bonded and non-hydrogen-bonded α,β-dihydrourocanic acids in the dielectric medium at the HF/6-31 + G(d,p) level with the SCIPCM. The energies were calculated from the structures optimized using the Onsager dielectric continuum model

ε[a]	ΔE_{dp}(HB)	ΔE_{dp}(nHB)	$\Delta\Delta E_{dp}$[c]	ΔpK_a[d]
2	307.9[b]	296.1	11.8	8.65
5	303.3	296.1	7.2	5.28
10	302.2	296.3	5.9	4.33
20	301.7	296.4	5.3	3.89
78.4	301.4	296.3	5.1	3.74

[a] Dielectric constant
[b] The energies of the Z form were obtained by using the geometry optimized at $\varepsilon = 5.0$
[c] The differences between ΔE_{dp}(HB) and ΔE_{dp}(nHB) values
[d] The amount pK_a changes on forming the LBHB in various dielectric media

deprotonation energy 7.2 kcal mol^{-1} larger, which corresponds to an increase of 5.3 units in the pK_a value. This result agrees very well with that from experiments [11]. Since the strength of the LBHB depends on the dielectric constant, so does the increase in the pK_a value.

Enzyme catalysis

In enzyme reactions, N-methylation of His 57 in α-chymotrypsin decreases its rate by 2×10^5 [35], and the Asp 102 mutagenesis of trypsin reduces its activity by 1×10^4 [36], which correspond to 7.2 and 5.5 kcal mol^{-1} of destabilization in terms of energy, respectively. These results were partly attributed to the loss of LBHBs in the active site in addition to the conformational rearrangements and multiple contributions of residues. Cassidy et al. [11] have reported that the strength of LBHB in the *N*-AcLF-CF_3 complex of chymotrypsin would be 11 kcal mol^{-1}, which agrees very well with the theoretical value at $\varepsilon = 5$ as shown in Table 5. This suggests that the α,β-dihydrourocanic acid can model the catalytic dyad of serine protease very well.

In serine protease, the LBHB in the protonated dyads increase the basicity of His 57 to abstract a proton from Ser 195 (p$K_a = 14$) [10, 11, 12]. The basicity of His 57 should be decreased later to give a proton from its conjugate acid form to a leaving group (p$K_a = 9$). This variation of the basicity can be achieved by changing the effective polarity of the active site. The small change in the location of the polar functional group in the active site can change the local dielectric constant, and the occupancy of the active site by substrate might reduce the polarity by blocking solvent access. Changing the value of ε between 2 and 10 induces a change of about 6 kcal mol^{-1} in the deprotonation energy as shown in Table 6. A relatively small variation in the polarity results in a fairly large change in the deprotonation energy, which is comparable to 4.4 units of the pK_a change. The pK_a value of His 57 is about 7 in serine proteases [11, 37, 38, 39, 40]. The pK_a value of His 57 in the dyad will be increased to 12.3 by forming LBHBs if the local dielectric constant in the active site of serine protease is 5, which agrees very well with the experimental value [11]. This pK_a value of the dyad will be further increased to 15.7 at $\varepsilon = 2$ and decreased to 11.3 at $\varepsilon = 10$. In fact the enzyme active site is not a dielectric continuum, and the local dielectric property is not homogeneous. There are also many specific interactions that may affect the enzymatic reactions, so the SCRF method cannot explain all the effects existing in the active site; therefore, these theoretical values may not be able to directly compare with the experimental results. However this study shows that even without including many other important aspects of the active site, the size and the change in the pK_a value of the dyad may be explained by local dielectric properties, which seems to be very important in understanding the enzyme mechanism.

The local dielectric constant in the active site of serine protease may be changed before and after substrate binding, or a proton abstraction from Ser 195 by His 57. The additional L-Leu residue in the *N*-AcLF-CF_3 complex of chymotrypsin increases the pK_a of the protonated dyad by about 1.2 [11]. This residue is nonpolar and probably reduces the local dielectric constant in the active site by rearranging polar functional groups away from the dyad. This means that reducing the polarity of the active site tends to increase the pK_a and the strength of the protonated dyad, which is consistent with the theoretical results in this study. Binding of a substrate makes the active site less polar and increases the pK_a of the dyad, which makes it possible to abstract a proton from Ser 195 [17]. After deprotonated Ser 195 attacks a substrate to form a tetrahedral intermediate, the active site may be reorganized again to bring polar functional groups closer. Consequently, the polarity will be increased and the deprotonation energy becomes smaller (the pK_a lower), so the protonated dyad can easily donate a proton to a leaving amino group. The theoretical estimates of the pK_a values vary between 16.2 and 11.2 depending on the dielectric constant. These values seem to be slightly overestimated, since many important specific interactions are not considered. However, it could be suggested that the local polarity of the active site of serine protease is important for the catalysis and it may be changed before and after the dyad abstracts a proton from Ser 195. In summary, the formation of LBHBs increases the basicity of the dyad, and the change in the polarity of the reaction center of the active site might help to abstract a proton from Ser 195 and to donate it to the leaving group. Both of them may play crucial roles in enzyme catalysis.

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