Protonation-State Dependence of Hydrogen Bond Strengths and Exchange Rates in a Serine Protease Catalytic Triad: Bovine Chymotrypsinogen A[†]

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ABSTRACT: Hydrogen-1 nuclear magnetic resonance spectroscopy was used to measure D/H fractionation factors and the temperature dependence of the rate of hydrogen exchange at two sites in the catalytic triad of chymotrypsinogen (hydrogen bond between aspartate-102 and histidine-57 and hydrogen bond between histidine-57 and serine-195) as a function of the protonation states of the constituent residues. Connectivities in one-dimensional spectra used to assign NMR data were collected at three pH values: pH 9, at which His-57 is neutral and Asp-102 is negatively charged; pH 3.5, at which His-57 is positively charged and Asp-102 is negatively charged; and pH 1, at which His-57 is positively charged and Asp-102 is neutral. The signal from $H^{\epsilon 2}$ of histidine-57 was assigned by reference to $^1H-^1H$ NOE connectivities at pH 3.5 to the previously assigned signals from the $H^{\epsilon 1}$ and $H^{\delta 2}$ of the same residue. The D/H fractonation factor, ϕ, for the hydrogen bond between Asp-102 and His-57 changed from $\phi = 2$ at pH 9 to $\phi = 0.4$ at pH 3.5. From studies of model systems, it may be concluded that a change of ϕ of this magnitude corresponds to a large increase in hydrogen bond strength. A signal from the hydrogen bond between Ser-195 and His-57 was detected only at the lower pH values studied. The D/H fractionation factor for this hydrogen bond was $\phi = 0.7$ at pH 3.5, indicative of a moderately strong interaction. Data obtained at pH 1 indicate that the hydrogen bond between Asp-102 and His-57 is weakened but that the hydrogen bond between His-57 and Ser-195 persists. The results are consistent with the hypothesis that changes in hydrogen bonding strength serve to lower barriers along the reaction coordinate in the catalytic mechanism. Large pH-dependent changes were found in the activation enthalpy ($\Delta H^{\ddagger}$) for exchange with protons from the solvent at the hydrogen bond between aspartate-102 and histidine-57: $\Delta H^{\ddagger}$ was approximately 10−12 kcal·mol^{-1} higher at pH 3.5 than at pH 1 or 9.

It has been proposed recently that strong hydrogen bonds (H-bonds)[1] play an important role in enzymatic catalysis in general (Gerlt & Gassman, 1993; Cleland & Kreevoy, 1994) and in catalysis by serine proteases (Gerlt & Gassman, 1993; Frey et al., 1994; Golubev et al., 1994) in particular. A basic feature of this hypothesis for serine proteases is that the H-bond between histidine-57 (His^{57}) and aspartate-102 (Asp^{102}) is weak in the resting enzyme but becomes strong in the transition state; this change serves to lower the energy of the transition state and thereby enhance catalysis (Frey et al., 1994; Golubev et al., 1994). Because weak H-bonds typically have energies in the range of 2−5 kcal·mol^{-1}, whereas strong H-bonds can have considerable covalent character with much higher bond energies (Hibbert & Emsley, 1990), such a mechanism for the enhancement of catalysis is attractive. Although limited experimental evidence for this proposal has come from an analysis of the shapes of NMR-derived titration curves for chymotrypsin (Golubev et al., 1994) and from comparison of the chemical shift of H and D in the H-bond between His^{57} and Asp^{102} in chymotrypsin at pH 3.5 (Frey et al., 1994), the hypothesis remains controversial (Warshel et al., 1995; Cleland & Kreevoy, 1995; Frey, 1995). Still earlier results from proton inventory studies have suggested that extensive changes in H-bonding accompany reactions catalyzed by serine proteases (Venkatasubban & Schowen, 1984; Schowen, 1988). This report contains new data relevant to these issues.

The gold standard for assaying the strengths of H-bonds in small molecules in the gas phase is ion cyclotron resonance (ICR). Alternatively, interatomic distances determined from single-crystal X-ray diffraction studies have been found to correlate well with H-bond strengths determined by ICR and other physical methods [see Hibbert and Emsley (1990) and references cited therein]. In X-ray structures of proteins, the presence of H-bonding is inferred from short distances between the centers of electron density corresponding to the heavy-atom H-bond donor and acceptor. However, because of the limited resolution of the X-ray structure of proteins, little can be said of quantitative strengths of H-bonds, although attempts in this direction have been made recently (Webber et al., 1989; Karpusas et al., 1990). H-Bonding also can be inferred from NMR data, from patterns of $^1H-^1H$ NOEs (Wagner et al., 1986), or from protection factors for hydrogen exchange (Wüthrich, 1986). Once again, it is

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[1] Abbreviations: H-bond, hydrogen bond; k_{ex}, observed rate of hydrogen exchange with the solvent; pK_a, negative logarithm of the acidity constant; ϕ, D/H fractionation factor.

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difficult quantify the strengths of H-bonds from such data.

In principle, strengths of H-bonds can be determined from shifts in the vibrational frequencies resulting from replacement of H by D, but this approach, which has been used with small molecules (Hibbert & Emsley, 1990), has not been applied successfully to the measurement of H-bond strengths in proteins. Correlations also have been found in small molecules between H-bond strengths and the magnitude of the difference in the chemical shift of the signal from the H-bonded atom when it is H (^{1}H NMR) and when it is D (^{2}H NMR). This was the method used by Frey and co-workers (Frey et al., 1994) with chymotrypsin. We were unable, however, to resolve the analogous ^{2}H NMR peak in spectra of chymotrypsinogen. The approach taken here to analyze H-bonding in chymotrypsinogen was to measure, as a function of the charge state of the catalytic triad, D/H fractionation factors (ϕ) and the temperature dependence of hydrogen exchange rates with solvent.

The D/H fractionation factor ϕ is defined as the equilibrium constant for the reaction

$$S{-}D + P_i{-}H \rightleftharpoons S{-}H + P_i{-}D \qquad (1)$$

where S represents the solvent, P_i the protein site, and H is protium and D is deuterium. D/H fractionation has been studied intensively in small molecules, but much less so in proteins. Hydrogens that participate in strong H-bonds in small model compounds typically exhibit D/H fractionation values (ϕ) significantly lower than 1.0 (Hibbert & Emsley, 1990). In the first extensive studies of D/H fractionation in proteins, it was found that backbone amide protons exhibited ϕ values between 0.3 and 1.8 in staphylococcal nuclease (Loh & Markley, 1994) and 0.63−1.4 in histidine-containing proteins (Bowers & Klevit, 1996). In staphylococcal nuclease, the average ϕ value for backbone amides at the surface of the protein was about 1.0, whereas the average ϕ for the protein as a whole was close to 0.8. This suggested that intramolecular H-bonding within the folded protein is stronger than protein−solvent or solvent−solvent interactions and that H-bonding is important in stabilizing the folded conformation of the protein. The smallest fractionation factors ($\phi = 0.3{-}0.6$) were for amides that donate an H-bond to side-chain carboxylates of aspartate or glutamate (Loh & Markley, 1994). One such couple is Thr^{120}···Asp^{77} ($\phi = 0.3$), an interaction that has been shown by mutagenesis to be critical in maintaining the stability of the protein (Hinck, 1993). Factors influencing D/H fractionation factors have been investigated recently by an *ab initio* computational approach (Edison et al., 1995a). Small calculated fractionation factors ($\phi \leq 0.6$), which correlated with strong H-bonding as characterized by short bond distances and orbital overlaps, emerged from model peptide systems that contained both charged interactions and extended (cooperative) hydrogen-bonded networks. The computations revealed that the strength of a given H-bond (and the value of the corresponding fractionation factor) can be tuned by making or breaking a H-bond at a distant site (Edison et al., 1995b). Recently, Mildvan and co-workers (Zhao et al., 1996) have correlated a measured D/H fractionation factor, determined by the NMR method employed here, with a thermodynamic measurement of H-bonding strength. Their results with Δ^5-3-ketosteroid isomerase correlated a D/H fractionation factor of $\phi = 0.34$ in an O−H···O hydrogen bond with a bond energy of 7.1 kcal mol^{-1}.

It is well-known that the reduced activity of serine protease zymogens arises from disordering of the substrate binding pocket rather than from disruption of the catalytic triad or Ser^{195}, His^{57}, and Asp^{102} (Freer et al., 1970). X-ray and NMR studies have indicated that the H-bonds of the catalytic triad are present in chymotrypsinogen (Fehlhammer et al., 1977; Markley, 1979) and other zymogens and have suggested that the Ser^{195}···His^{57} H-bond in the zymogen is stronger than that in the enzyme in the absence of substrate. In addition, the pK_a value for addition of the first proton to the triad is higher in chymotrypsinogen (7.3) than in chymotrypsin (6.1) (Markley & Ibañez, 1978), and closer to its pK_a in transition state model complexes (Porubcan et al., 1979; Liang & Ables, 1987; Zhong et al., 1995). It was for these reasons that we chose bovine chymotrypsinogen A for our first investigations of D/H fractionation in serine proteinases.

The textbook reaction mechanism for serine proteinases has His-57 serving as a general base and accepting a proton from Ser-195 as the reaction proceeds from the Michaelis complex to the first transition-state tetrahedral complex. The tetrahedral species in peptide hydrolysis has never been trapped and hence cannot be studied as such. Our goal was to investigate changes in the strength of H-bonding between His-57 and Asp-102 between the Michaelis and tetrahedral complexes. Our approach was to model this change by using a change in pH to effect the change in protonation. The implicit assumption was that, since major changes in the structure of the protein do not occur over this pH range (pH 9−3.5) as revealed by NMR (Markley & Ibañez, 1978) studies, comparison of the results would reveal local changes resulting from protonation of His-57. This same assumption is invoked in analyzing spectroscopically-determined titration curves to obtain pK_a values of individual groups in proteins which have proved valuable in interpreting the pH dependence of catalytic rates. Data obtained at pH 9 were taken to model the state of the catalytic triad in the Michaelis complex, and data obtained at pH 3.5 were assumed to model the postulated tetrahedral intermediate in which a proton has been transferred from Ser^{195} to His^{57}. By the Hammond postulate (Jencks, 1969), the tetrahedral intermediate should be much closer to the transition state than the Michaelis complex so that the results obtained at pH 3.5 can be used to approximate the state of these interactions in the rate-determining step. For completeness, data were collected also at pH 1 where the catalytic triad has accepted a second proton; this state has no physiological relevance. The spectral data yielded D/H fractionation factors and the temperature dependence of the exchange rates of the hydrogens participating in H-bonds in the catalytic triad. These values were found to be highly dependent on the charge state of the catalytic triad.

MATERIALS AND METHODS

^{1}H NMR spectra were obtained at 750 MHz on the Bruker DMX 750 spectrometer in the National Magnetic Resonance Facility at Madison. Chymotrypsinogen A was purchased from Sigma (St. Louis, MO, lot 49F8005). All chymotrypsinogen solutions contained 0.1 M KCl. Samples were titrated to the desired pH values (combination glass electrode readings) with KOH or HCl.

Exchange rates with the solvent for the hydrogens giving rise to the low-field signals were determined from line-width

measurements at all three pH values over the temperature range 1−19 °C. Activation enthalpies ($\Delta H^{\ddagger}$) were estimated from the temperature dependence of the rates; activation free energies ($\Delta G^{\ddagger}$) were calculated from the exchange rates on the basis of reaction rate theory. Activation entropies ($\Delta S^{\ddagger}$) were calculated from the $\Delta H^{\ddagger}$ and $\Delta G^{\ddagger}$ values by the equation, $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$. Intrinsic exchange rates for $H^{\delta 1}$ and $H^{\epsilon 2}$ at the three pH values were calculated (Wagner & Wüthrich, 1979) on the basis of experimental pK_a values of the two *N*-methylhistidines: $N^{\delta 1}-CH_3$, pK_a = 6.8; $N^{\epsilon 1}-CH_3$, pK_a = 6.1 (Markley, 1979). Protection factors were calculated from ratios of the intrinsic to observed exchange rates. The exchange rates and their temperature dependence at pH 3.5 were also measured by following the repopulation of each site ($H^{\delta 1}$ and $H^{\epsilon 2}$ of His-57) from bulk solvent following selective saturation of each of the two signals; the exchange rates obtained at 1 °C by this approach were equivalent, within experimental error, to those determined from line widths; the activation energies, however, were larger by a factor of 2.

Fractionation factors for chymotrypsinogen at 3 °C were determined by measuring peak intensities (relative to non-exchangeable high-field methyl peaks) in a series of samples containing different H_2O/D_2O solvent compositions at the same three pH values. Samples used for D/H fractionation factor measurements were prepared as follows. For each pH studied, two stock solutions of 2 mM bovine chymotrypsinogen A in 0.1 M KCl were prepared in 5-mL volumetric flasks: one in H_2O and one in D_2O (99.9%, Isotec, Inc., Miamisburg, OH). Each pair of stock solutions was titrated to the pH of a given study; then appropriate volumes from each solution were micropipetted into 5 mm NMR tubes (528-PP, Wilmad, Buena, NJ) to yield samples of 400 μL. These were mixed and then stored on ice until the spectra were taken. The H content of the stock solution in D_2O was assayed by reference to the intensity of the residual water peak before and after adding a measured small quantity of H_2O. The short exchange lifetimes determined above assured that each sample was at D/H exchange equilibrium. A 1-1 pulse sequence (Hore, 1983) was used to minimize saturation of the water signal; however, differential saturation of the water peak was observed in samples at different H_2O/D_2O compositions. This effect on the water signal was quantified, and an appropriate correction factor was applied to the intensity measurements of the exchangeable proton NMR signals from the protein. Because the recycle times used in these experiments were fast compared to the relaxation time for the solvent, it was necessary to correct the measured peak intensities for effects of saturation transfer. This correction took into account the dependence of the relaxation time of the solvent on the deuterium/protium ratio (Abragam, 1961). The correction factor was determined by measuring the apparent fractionation factor of the solvent and then normalizing it to unity. The quadratic contribution to the apparent fractionation factor of water at low deuterium concentration is small and was neglected. Fractionation factors were determined by fitting the data to the linear equation (Loh & Markley, 1994):

$$(yC)^{-1} = [\phi(1 - X)/X] + 1 \qquad (2)$$

where y is the signal intensity, C is a normalization factor, and the slope of the line is the D/H fractionation factor (ϕ).

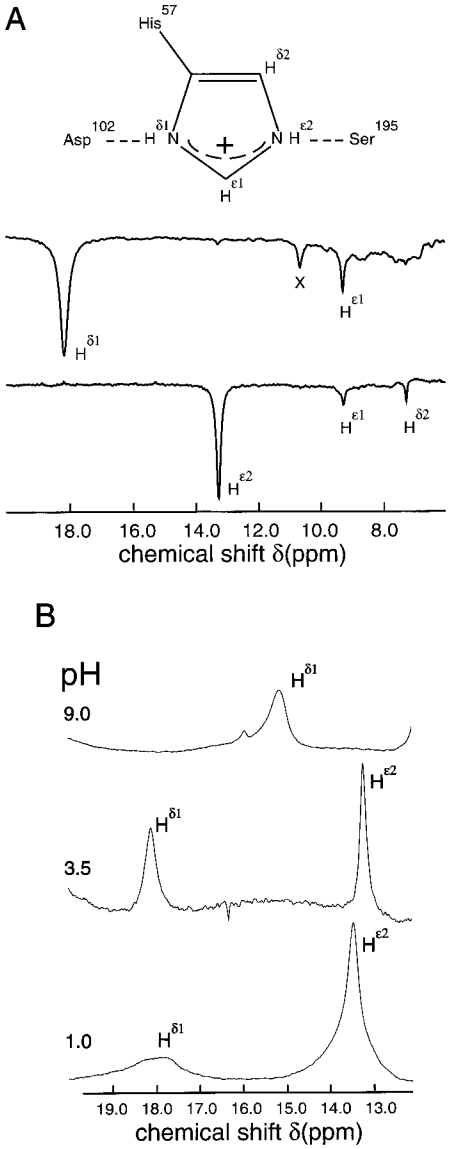

FIGURE 1: Low-field region of ^{1}H NMR spectra of 2 mM chymotrypsinogen in H_2O at 1 °C; a 1-1 pulse sequence (Hore, 1983) was used. (A) Truncated, driven NOE difference spectra (Wagner & Wüthrich, 1979) of low-field ^{1}H NMR signals from histidine-57 at pH 3.5. The largest peak in each trace corresponds to the resonance that was selectively saturated for 50 ms; signals from ring protons of histidine-57 excited by cross-relaxation (NOE) are labeled according to the inset; peak "X" may be the signal from the nearby backbone amide proton of histidine-57. (B) Comparison of spectra of chymotrypsinogen at pH 9, pH 3.5, and pH 1. Signals from the imidazole hydrogen bonds of histidine-57 are labeled according to the inset in panel A.

RESULTS

^{1}H NMR spectra of chymotrypsinogen, collected with nonsaturating solvent suppression at 750 MHz in H_2O at pH 3.5, confirmed the presence of signals previously reported at 18 ppm (Robillard & Shulman, 1974; Liang & Ables, 1987) and 13 ppm (Markley, 1978; Liang & Ables, 1987). These were assigned to protons on His^{57} by means of one-dimensional, truncated-driven nuclear Overhauser experiments obtained at pH 3.5 (Figure 1A): an NOE connectivity between the peak at 18 ppm and the previously-assigned $H^{\epsilon 1}$ signal of His^{57} (Markley & Ibañez, 1978) confirmed the assignment of the 18 ppm peak to $H^{\delta 1}$; a connectivity between the peak at 13 ppm and the signal assigned to $H^{\epsilon 1}$ provided a new assignment of the 13 ppm peak to the $H^{\epsilon 2}$

Table 1: NMR-Derived Parameters for Hydrogen Bonds in the Catalytic Triad of Bovine Chymotrypsinogen A

H of His-57 in catalytic triad obsd by NMR (boldface type)	pH at 20 °C (glass electrode)	chemical shift at 1−5 °C (ppm)	exchange rate, k_{ex} at 1 °C ($s^{-1} \times 10^{-3}$)	calcd intrinsic exchange rate ($s^{-1} \times 10^{-3}$)	protection factor	activation free energy for exchange, $\Delta G^{\ddagger}$ (kcal·mol^{-1})	activation enthalpy for exchange $\Delta H^{\ddagger}$ (kcal·mol^{-1})	activation entropy for exchange, $\Delta S^{\ddagger}$ (eu)	D/H fractionation factor 5 °C (ϕ)
Asp$^{(-)}$···**H**$^{\delta 1}$−His$^{(0)}$	9	15.2	3.52	10.7	3.0	12.1 ± 0.3	−3.4 ± 0.4	−54 ± 3	2.0 ± 0.1
Asp$^{(-)}$... **H**$^{\delta 1}$−His$^{(+)}$	3.5	18.1	0.952	8.72	9.2	12.4 ± 0.1	10.7 ± 0.3	−6 ± 1	0.40 ± 0.02
Asp$^{(0)}$···**H**$^{\delta 1}$−His$^{(+)}$	1	18.0	3.94	8.72	2.2	11.9 ± 0.3	−0.5 ± 0.4	−44 ± 2	1.4 ± 0.2
His$^{(+)}$−**H**$^{\epsilon 2}$···Ser$^{(0)}$	3.5	13.2	0.383	1.74	4.5	13.1 ± 0.2	3.0 ± 0.4	−35 ± 2	0.69 ± 0.01
His$^{(+)}$−**H**$^{\epsilon 2}$···Ser$^{(0)}$	1	13.4	1.23	1.74	1.4	12.6 ± 0.3	−0.3 ± 0.3	−45 ± 2	0.54 ± 0.03

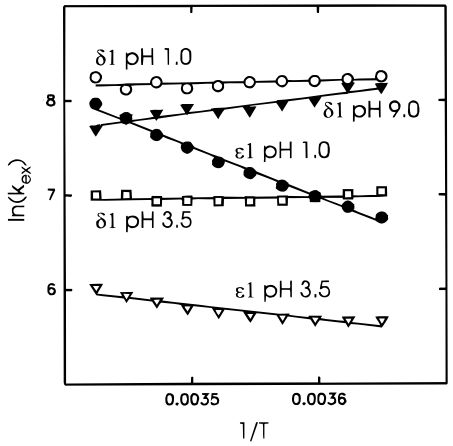

FIGURE 2: Temperature dependence of the hydrogen exchange rates of the imidazole NHs of histidine-57 ($H^{\delta 1}$ is the atom hydrogen bonded to aspartate-102; $H^{\epsilon 2}$ is the atom hydrogen-bonded to serine-195) determined at three pH values: pH 1, pH 3.5, and pH 9. Symbols: (○), $H^{\delta 1}$ at pH 1.0; (●), $H^{\epsilon 1}$ at pH 1.0; (▼), $H^{\delta 1}$ at pH 9.0; (□), $H^{\delta 1}$ at pH 3.5; (▽), $H^{\epsilon 1}$ at pH 3.5.

of His^{57}; the additional connectivity to the peak at 13 ppm was assigned to the $H^{\delta 1}$ of His^{57}. Assigned spectra obtained at the three pH values are presented in Figure 1B.

Figure 2 shows an analysis of the temperature dependence of the exchange rates determined at the three pH values. The activation enthalpies, derived from the temperature dependence of the exchange rates, were small for both the $H^{\delta 1}$ and $H^{\epsilon 1}$ hydrogens at pH 1, slightly endothermic for $H^{\epsilon 1}$ and strongly endothermic for $H^{\delta 1}$ at pH 3.5, and slightly exothermic for $H^{\delta 1}$ at pH 9 (Table 1). The data for both $H^{\delta 1}$ and $H^{\epsilon 1}$ at pH 3.5 show slight curvature (Figure 2); this may result either from the temperature dependence of the heat capacity for activation or from the existence of more than one activated process. The exchange rates, activation energies for exchange, and derived thermodynamic parameters are summarized in Table 1.

Representative ^{1}H NMR spectra of 2 mM chymotrypsinogen at pH 3.5 as a function of the mole fraction H_2O/D_2O are shown in Figure 3A. The D/H fractionation factors (Table 1) were derived from the slopes of the plots shown in Figure 3B.

DISCUSSION

The proton hydrogen bonded between Asp^{102} and His^{57} exhibited relatively low protection factors for exchange at all three pH values studied (Table 1); these are many orders of magnitude smaller than protection factors observed for backbone amides in stable, solvent-excluded regions of proteins (Roder, 1989). These experimental results are consistent with earlier observations that the ^{1}H NMR signal from His^{57} $H^{\delta 1}$ could only be resolved at low temperatures (Robillard & Shulman, 1974; Markley, 1978) where exchange is slowed, but the finding is remarkable because the Asp^{102}−His^{57} diad is shielded from solvent (Fehlhammer et al., 1977). Although $\Delta G^{\ddagger}$ for exchange is relatively constant with pH, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ show large compensating changes that depend on the protonation state of His^{57}. The highest activation energies for exchange at the two sites taken together are found at the pH (3.5) where the charge state of the catalytic triad is that of the tetrahedral intermediate.

The experimental D/H fractionation factors (ϕ) show large changes dependent on the charge state of the catalytic triad (Table 1). The value of ϕ for $H^{\delta 1}$ of His^{57} decreases from about 2 at pH 9 to 0.4 at pH 3.5 and then increases to 1.4 at pH 1. These results suggest that the strength of the H-bond (bond enthalpy) is strongest in the singly protonated form of the catalytic triad. Although no data are available for the H-bond that Ser^{195} donates to His^{57} at pH 9, the results indicate that the H-bond between His^{57} $H^{\delta 1}$ and Ser^{195} O^{γ} is relatively strong at pH 3.5 (ϕ value of 0.7). This strong H-bond persists at pH 1 ($\phi = 0.5$).

Thus, both the activation energy measurements for H-exchange with solvent and, more importantly, the D/H fractionation factor measurements support the hypothesis (Frey et al., 1994; Golubev et al., 1994) that a marked increase in H-bonding strength (enthalpy) accompanies the addition of a proton to the Asp^{102}−His^{57} catalytic diad, as occurs on going from the resting enzyme (or Michaelis complex) to the tetrahedral intermediate or nearby transition state. In addition, the present results indicate that the imidazolium of His^{57} donates a moderately strong H-bond to the hydroxyl oxygen of Ser^{195}. Thus, the stabilization cannot be attributed just to a change in the energy of the H-bond between Asp^{102} and His^{57}. Instead, the effect appears to be explained better in terms of a change in the energy of the cooperative network of H-bonds that includes the catalytic triad, but extends to neighboring residues as suggested by the X-ray structure (Wang et al., 1985) of this region of the protein (Figure 4).

Attention has been given recently to correlations between the magnitudes of chemical shifts of protons in H-bonds and H-bond strengths (Tobin et al., 1995) as found in small molecule model systems. However, the chemical shift of $H^{\delta 1}$ of His^{57} of chymotrypsinogen (Table 1) is nearly the same at pH 3.5 (18.1 ppm), where it participates in a network of strong hydrogen bonds, as characterized by its large activation energy for exchange ($\Delta H^{\ddagger} = 11.9$ kcal·mol^{-1}) and low fractionation factor ($\phi = 0.4$), and at pH 1 (18.0 ppm), where the strength of its H-bond to Asp^{102} is weakened, as reflected by the low activation barrier for exchange ($\Delta H^{\ddagger} \cong 0$) and large fractionation factor ($\phi = 1.4$). Thus, one may

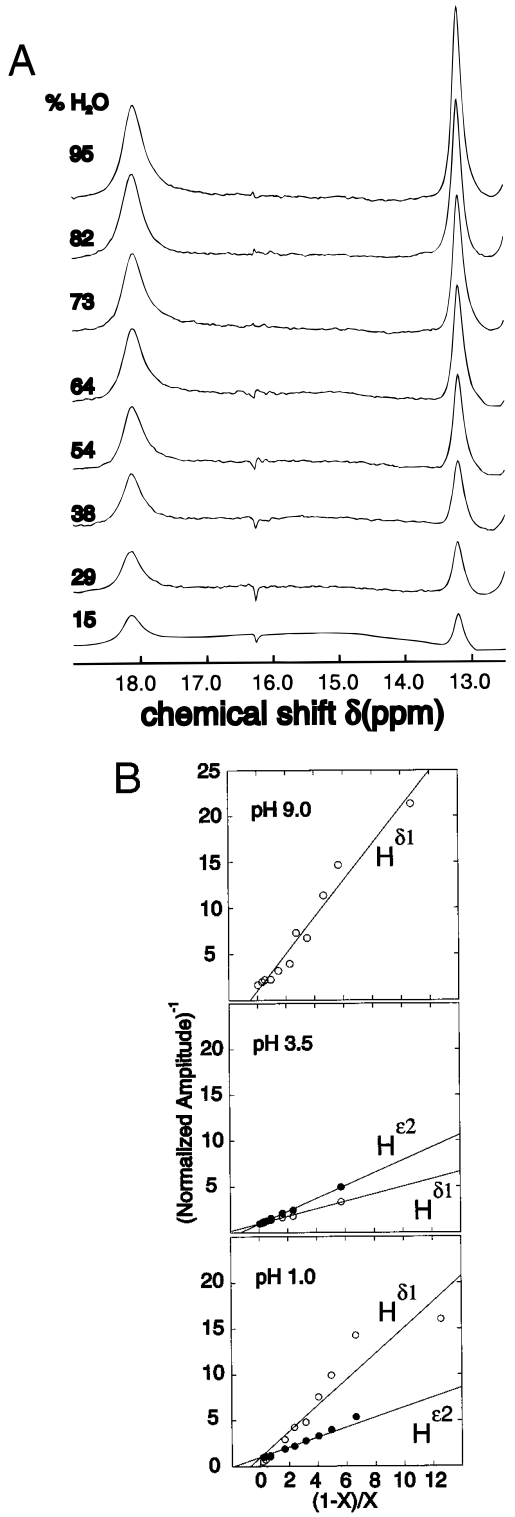

FIGURE 3: Measurement of D/H fractionation at the catalytic triad of chymotrypsinogen. (A) Sample ^{1}H NMR spectra of 2 mM chymotrypsinogen as a function of the mole fraction H_2O/D_2O at pH 3.5. (B) Plots of the normalized ^{1}H NMR signal intensity (yC) as a function of the mole fraction H_2O (X) according to the equation (Loh & Markley, 1994): $(yC)^{-1} = [\phi(1 - X)/X] + 1$, where y is the signal intensity, C is a normalization factor, and the slope of the line is the D/H fractionation factor (ϕ).

not expect to find a simple correlation between the proton chemical shift and H-bond strength in more complex protein systems.

One may question the suitability of any ground-state analogue as models for the transition state of serine proteinases (Schowen, 1988) or other enzymes. While it is true

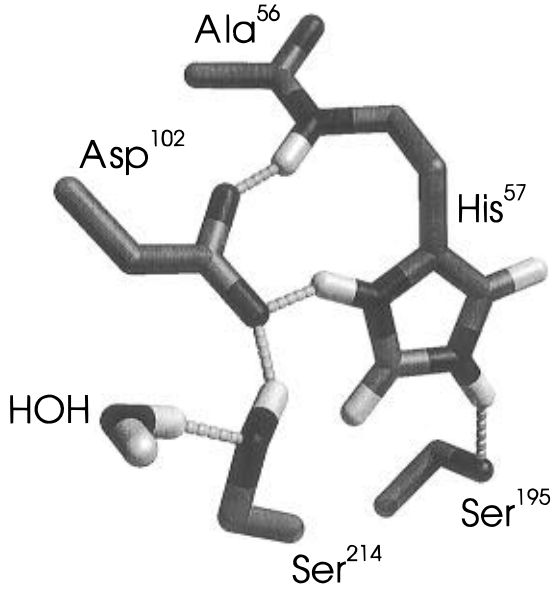

FIGURE 4: Scheme showing the network of hydrogen bonds involving the catalytic triad of chymotrypsinogen as identified from the X-ray structure at 1.8 Å (Wang et al., 1985).

that the transition state, which has not been trapped, is the species one ideally would like to study in order to understand catalytic mechanisms, one can probe certain probable characteristics of the transition state by examining stable species. Here we are asking the limited question of whether the strengths of the H-bond interactions between members of the catalytic triad, some distance removed from the site of chemical bond formation, depend on their protonation state, which is postulated to change as the catalytic mechanism proceeds. At the simplest level, one can ask, as was done here, whether such changes exist in the absence of substrate binding. At a higher level, one can ask about H-bonding under conditions where a transition-state analog inhibitor is bound. The results of such an investigation, D/H fractionation in subtilisin−inhibitor complexes (Halkides et al., 1996), confirm the idea that a change in the strength of H-bonding within the catalytic triad accompanies the formation of the transition state. Further studies of the type recently carried out by Zhao et al. (1996) and Bowers and Klevit (1996) should build a firmer basis for the interpretation of D/H fractionation factors in terms of bond energies.

It will be of interest to extend these studies to transition-state models of other members of the chymotrypsin and subtilisin families of serine proteases and to wheat serine carboxypeptidase II, which has a different geometry for the Asp···His hydrogen bond in the catalytic triad (Liao et al., 1992). It will be important to determine, in addition, whether strong H-bonding occurs within the oxyanion hole (Gerlt & Gassman, 1993), as expected from considerations of cooperative H-bonding (Edison et al., 1995b). An intriguing result reported here is the low degree of protection against H-exchange within the catalytic triad. The possible importance of this in enzyme catalysis, e.g., as a mechanism for protonating the leaving group, bears future scrutiny; this feature may explain evidence for multiple protons being in flight in the rate-determining step (Schowen, 1988; Kresge, 1973). The new methods described here should be applicable to investigations of H-bonding in a wide range of other enzyme systems.

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