# A New Concept for the Mechanism of Action of Chymotrypsin: The Role of the Low-Barrier Hydrogen Bond†

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ABSTRACT: The basicities of the diad H57−D102 at $N^{\epsilon 2}$ in the tetrahedral complexes of chymotrypsin with the peptidyl trifluoromethyl ketones (TFK) *N*-acetyl-L-Leu-DL-Phe-$CF_3$ and *N*-acetyl-DL-Phe-$CF_3$ have been studied by $^1$H-NMR. The protons bridging His 57 and Asp 102 in these complexes are engaged in low-barrier hydrogen bonds (LBHBs). In $^1$H-NMR spectra at pH 7.0, these protons appear at $\delta$ 18.9 and 18.6 ppm, and the p$K_a$s of the diads are 12.0 ± 0.2 and 10.8 ± 0.1, respectively. The difference indicates that removal of leucine from the second aminoacyl site $S_2$ of chymotrypsin weakens the LBHB and decreases the basicity of the H57−D102 diad relative to the case in which $S_2$ is occupied by leucine. Consideration of the available structural data on chymotrypsin and other serine proteases, together with the high p$K_a$s of the hemiketals formed with TFKs, suggests that LBHB formation in catalysis arises through a substrate-induced conformational transition leading to steric compression between His 57 and Asp 102. Because the N−O distance in the LBHB is shorter than the Van der Waals contact distance, the LBHB is proposed to stabilize the tetrahedral intermediate through relief of steric strain between these residues. In this mechanism, substrate-induced steric compression within the diad increases the basicity of $N^{\epsilon 2}$ in His 57, making it a more effective base for abstracting a proton from Ser 195 in the formation of the tetrahedral intermediate. The values of p$K_a$ for $N^{\epsilon 2}$ in TFK adducts lie between those of Ser 195 (p$K_a \approx 14$) and the leaving group in tetrahedral adducts (p$K_a \approx 9$), making $N^{\epsilon 2}$ of the H57−D102 diad strong enough as a base to abstract the proton from Ser 195 in tetrahedral adduct formation but not so strong that its conjugate acid cannot protonate the leaving group. According to this theory, the "normal" p$K_a$ of His 57 in free chymotrypsin arises from the use of part of the stabilization energy provided by the LBHB to drive the conformational compression required for its formation. In catalysis, the energy for conformational compression is supplied by the binding of remote portions of the substrate, including the side chains of $P_1$ and $P_2$.

Low-barrier hydrogen bonds (LBHBs),[1] also described as short, strong hydrogen bonds, are exceptionally strong under certain conditions and can be formed under special circumstances (Hibbert & Emsley, 1990). They have been postulated to stabilize intermediates in enzymatic catalysis of enolization (Cleland & Kreevoy, 1994; Gerlt & Gassmann, 1993). Spectroscopic evidence has been cited in support of the participation of an LBHB between His 57 and Asp 102 of chymotrypsin in stabilizing a tetrahedral intermediate formed between Ser 195 and the carbonyl group of a substrate according to Scheme 1 ($0.5 \leq |y| \leq 1.0$; Frey et al., 1994). The idea that hydrogen bonds of this type can exist in proteins or participate in catalysis has been criticized (Guthrie & Kluger, 1993; Warshel et al., 1995).

Peptidyl trifluormethyl ketones (TFKs) form stable hemiketals at the active site of chymotrypsin, in which the 3-hydroxy group of Ser 195 is added to the carbonyl group of the TFK (Imperiali & Abeles, 1986; Liang & Abeles, 1987; Brady et al., 1990). These adducts are good models for the metastable tetrahedral intermediate in the mechanism of Scheme 1. The tetrahedral complex of *N*-Ac**LF**-$CF_3$ with chymotrypsin contains the LBHB postulated for the tetrahedral intermediate, as indicated by its low-field proton NMR signal at $\delta$ 18.7 ppm for the H57−D102 diad, which remains protonated at pHs as high as 9.5 (Liang & Abeles, 1987). Moreover, the p$K_a$ of the hemiketal hydroxyl group is <4.0 (Brady et al., 1989). These properties can be explained by the effects of the LBHB in stabilizing the complex (Frey et al., 1994).

In this paper we update the NMR properties of the LBHBs in complexes of chymotrypsin with TFKs, we report p$K_a$ values of the H57−D102 diads, and we compare them with the normal p$K_a$ for histidylpeptides. We explain the differences in terms of a new concept for the role of the LBHB in facilitating the formation of tetrahedral intermediates or tetrahedral-like transition states.

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[1] Abbreviations: NMR, nuclear magnetic resonance; LBHB, low-barrier hydrogen bond; TFK, peptidyl trifluoromethyl ketone; *N*-Ac**LF**-$CF_3$, *N*-acetyl-L-leucine-DL-phenylalanine trifluoromethyl ketone; *N*-Ac**F**-$CF_3$, *N*-acetyl-DL-phenylalanine trifluoromethyl ketone; Cht, chymotrypsin; DIP, diisopropylphosphoryl; MIP, monoisopropylphosphoryl; BPTI, bovine pancreatic trypsin inhibitor; SBTI, soybean trypsin inhibitor; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

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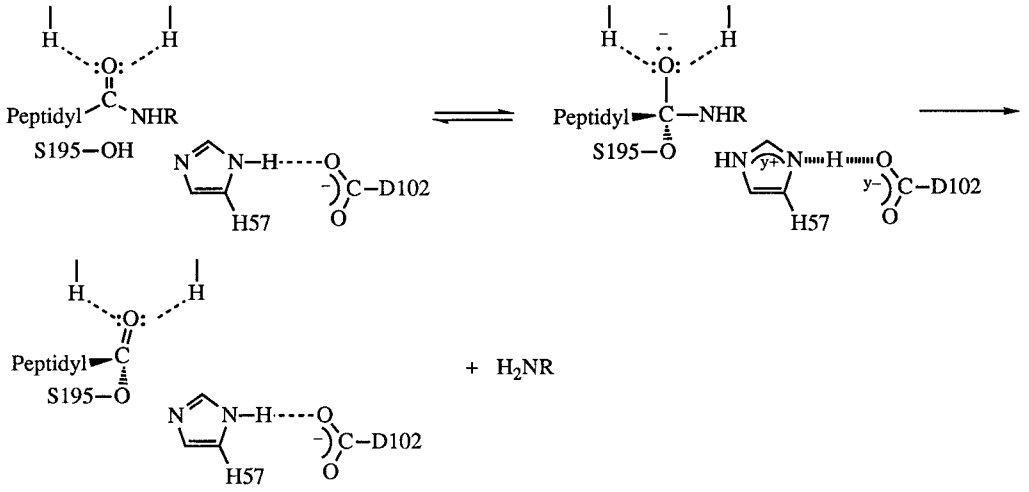
Scheme 1




## EXPERIMENTAL PROCEDURES

α-Chymotrypsin (type II, 3× crystallized from bovine pancreas) and CHES were obtained from Sigma and used as supplied. Peptidyl trifluoromethyl ketones were synthesized as described elsewhere (Imperiali & Abeles, 1986). Acetonitrile-$d_3$, 99% $D_2O$, and CAPS were obtained from Aldrich. Other chemicals were purchased from commercial suppliers.

Solutions of ~2 mM chymotrypsin were prepared with TFKs at pHs ranging from 7 to 12.5 in 0.5 M buffers (CAPS, CHES, and phosphate). Peptidyl trifluormethylketones were dissolved in acetonitrile and added in $\mu$L aliquots to the chymotrypsin samples so that the solutions contained ≤4% acetonitrile and 1.5 equiv of *N*-Ac**LF**-$CF_3$ or 2.78 equiv of *N*-Ac**F**-$CF_3$ relative to chymotrypsin, as well as 0.04% w/v of sodium 3-(trimethylsilyl)-1-propanesulfonate in $D_2O$ as an internal standard. The pH of each sample was measured at 5 °C.

$^1$H NMR spectra were acquired on a Bruker DMX 500 MHz spectrometer using a nonsaturating water signal suppression program at 5 °C (Hore, 1983). The probe was tuned for each sample, and the 90° pulse was calibrated. The water signal was suppressed using a 1−1 pulse sequence program. Spectral conditions were 8K time domain points and 8000 scans.

The data were processed at a Silicon Graphics workstation using the Felix (version 2.3) software package. The exponential line broadening was set to 20 Hz. Baselines were corrected with a fifth-order polynomial. The low-field signals were integrated by cutting and weighing. The relative areas of the peaks at 18.9 ppm (*N*-Ac**LF**-$CF_3$) or 18.6 ppm (*N*-Ac**F**-$CF_3$) and 15 ppm (neutral His 57) were determined. For the titration of the complex containing *N*-Ac**LF**-$CF_3$, the enzyme was not fully saturated with the inhibitor under the experimental conditions, so that the relative peak areas were corrected for uncomplexed enzyme.

## RESULTS

Shown in Figure 1 are representative $^1$H-NMR signals of the downfield protons corresponding to the bridging proton in the H57−D102 diad in the tetrahedral adduct formed between Ser 195 of chymotrypsin and *N*-Ac**LF**-$CF_3$ at various pHs. The low-field proton (LBHB) resonates at 18.9 ppm at pH 7−8 (135 Hz bandwidth), and it is still present in a slightly broadened form at pH 11.9. This signal is at a lower field and is narrower than that for the protonated diad in free chymotrypsin (18.3 ppm, 230 Hz at pH 4.30, and a broadened 17.6 ppm signal at pH 7.22; Zhong et al., 1995). At higher pHs, the decrease of the signal at 18.9 ppm and the growth of a new signal at 15 ppm (140 Hz bandwidth) indicated the ionization of the diad ($N^{\epsilon 2}$). The new signal was indistinguishable from that for the H57−D102 diad in free chymotrypsin at high pH (15 ppm at pH 10.34, 144 Hz; Zhong et al., 1995). The lower field position of the signal for the LBHB than that reported earlier (18.7 ppm) is attributed to the fact that an internal reference was employed

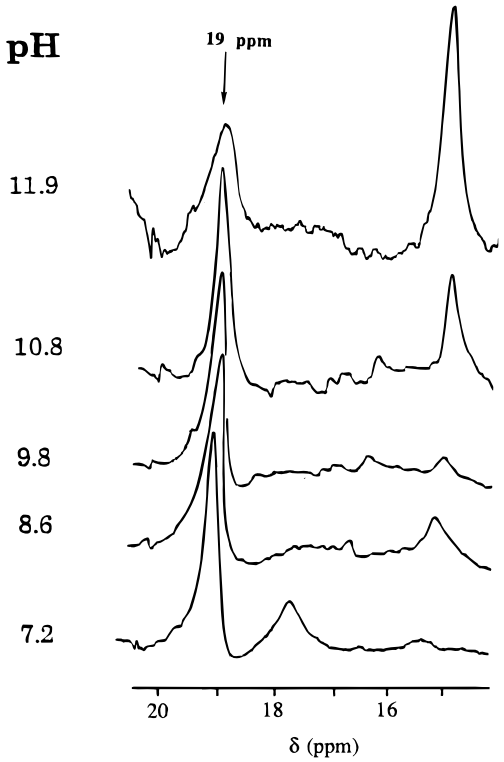



FIGURE 1: Downfield NMR spectra of the tetrahedral addition complex formed between chymotrypsin and *N*-Ac**LF**-$CF_3$ at various pH values. Representative spectra for samples between pH 7.2 and 11.9 are shown. The compositions of the solutions and conditions for data accumulations are given in Experimental Procedures. The signal at 18.9 ppm is that of the protonated H57−D102 diad in the tetrahedral complex formed between *N*-Ac**LF**-$CF_3$ and chymotrypsin. The 15 ppm signal that grows at higher pHs at the expense of the 18.9 ppm signal is that of the unprotonated diad in free chymotrypsin. The minor signal at 17.6 ppm in the sample at pH 7.2 is that of the protonated diad in free chymotrypsin.

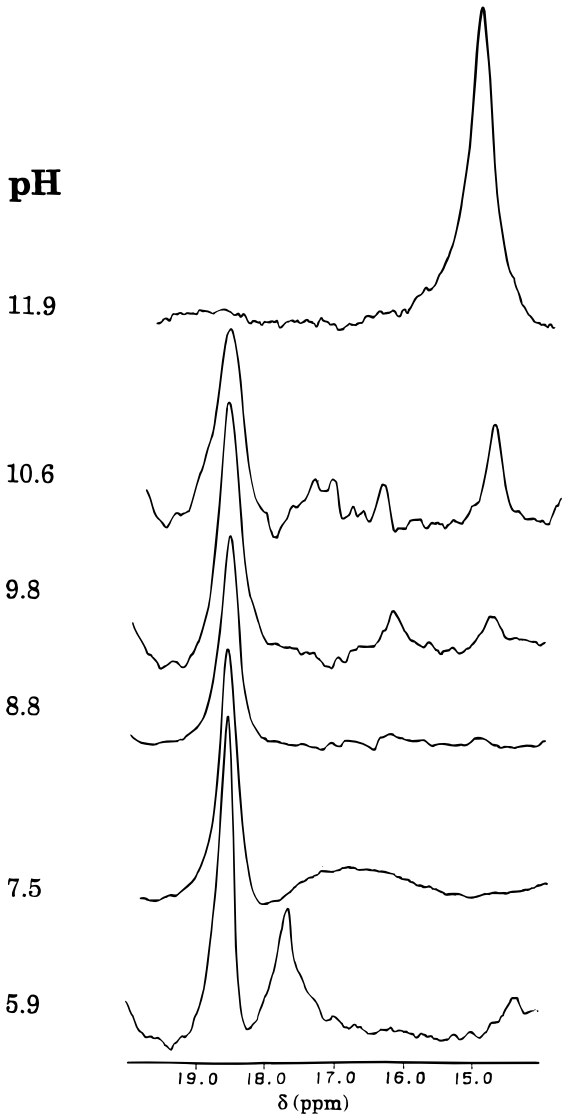



FIGURE 2: Downfield NMR spectra of the tetrahedral addition complex formed between chymotrypsin and *N*-Ac**F**-$CF_3$ at various pH values. Representative spectra for samples between pH 7.2 and 11.9 are shown. The compositions of the solutions and conditions for data accumulations are given in Experimental Procedures. The signal at 18.9 ppm is that of the protonated H57−D102 diad in the tetrahedral complex formed between *N*-Ac**LF**-$CF_3$ and chymotrypsin. The 15 ppm signal that grows at higher pHs at the expense of the 18.9 ppm signal is that of the unprotonated diad in free chymotrypsin. The minor signal at 17.6 ppm in the sample at pH 5.9 is that of the protonated diad in free chymotrypsin.

in the present work, whereas an external reference was used previously (Liang & Abeles, 1987).

The reaction of *N*-Ac**F**-$CF_3$ with chymotrypsin also produces a tetrahedral complex that displays a low-field $^1$H-NMR signal, 18.6 ppm at pH 8 (150 Hz bandwidth). This signal is narrower and at lower field than that for the protonated diad in free chymotrypsin. Representative spectra showing the low-field signals for the *N*-Ac**F**-$CF_3$ complex at various pHs are shown in Figure 2. As in the case of *N*-Ac**LF**-$CF_3$, the intensity of the 18.6 ppm signal decreases and the signal at 15 ppm corresponding to free chymotrypsin grows concomitantly with increasing pH.

In earlier work, a lower limit of 10 was set for the p$K_a$ of the diad in the complex of chymotrypsin with *N*-Ac**LF**-$CF_3$ (Liang & Abeles, 1987). Intensities of the 18.9 and 18.6 ppm signals from this complex and that of *N*-Ac**F**-$CF_3$ are plotted against pH in Figure 3. The intensity of the signal

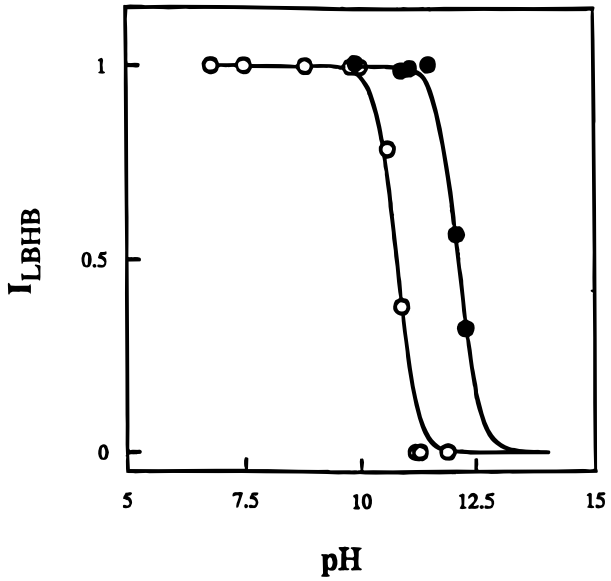



FIGURE 3: pH titrations of the low-field $^1$H NMR signals for the tetrahedral complexes of *N*-Ac**LF**-$CF_3$ and *N*-Ac**F**-$CF_3$ with chymotrypsin. The low-field signals for the complexes were obtained and integrated as described in the Experimental Procedure. The signal intensities were corrected for the amount of uncomplexed enzyme and plotted against pH. Open circles are data for *N*-Ac**F**-$CF_3$, and closed circles are for *N*-Ac**LF**-$CF_3$. The titration data do not fit an equation for a single ionization. Therefore they are fitted to the equation $I_{LBHB} = 1/(1 + K/[H^+]^2)$. The fits indicate the cooperative dissociation of at least two protons, one of which results from deprotonation of the H57−D102 diad. The source of the second proton is discussed in the text.

for the *N*-Ac**F**-$CH_3$ complex persists at pHs as high as 10 and then falls off sharply at higher pHs. Analogous data for the 18.9 ppm signal corresponding to the complex with *N*-Ac**LF**-$CF_3$ display a sharp decrease in intensity at pHs above 11.5. The results in Figure 3 are representative of data obtained in two or three experiments for each complex and are reproducible. His 57 in the complex of chymotrypsin with *N*-Ac**LF**-$CF_3$ is to the authors' knowledge the most basic histidine residue so far reported in an enzyme.

Inspection of the titration curves in Figure 3 allows two conclusions regarding the ionizations of the tetrahedral adducts of TFKs with chymotrypsin. First, the curves describe complex proton dissociation behavior that appears to be similar for the two TFKs, except for being displaced on the abscissa by about 1.5 pH units. The titration data are highly cooperative and, in Figure 3, are fitted to the equation $I_{LBHB} = 1/(1 + K/[H^+]^2)$, where $I_{LBHB}$ is the integrated intensity of the low-field signal relative to the unionized form at pH 8. Second, regardless of the molecular basis for the cooperative multiproton ionization, the p$K_a$s must be higher than the break-points in the titration curves. Data from the complex with *N*-Ac**F**-$CF_3$ fitted as a two-proton dissociation give a p$K_a$ of 10.8 for the diad, and a repeat of the titration gives a p$K_a$ value of 10.7. Fitting of the titration data for the tetrahdral complex with *N*-Ac**LF**-$CF_3$ gives a p$K_a$ of 12.2 in one experiment and 11.8 in a second. The p$K_a$ for the complex with *N*-Ac**LF**-$CF_3$ is less well-determined because of being so high that data could not be obtained at pHs much above the p$K_a$.

The steep titration curves do not result from irreversible alkaline denaturation. In the first place, the steep curves are observed with both *N*-Ac**LF**-$CF_3$ above pH 12 and *N*-Ac**F**-$CF_3$ above pH 10. Second, data were routinely acquired for the NMR spectra over a period of 2 h; however, data

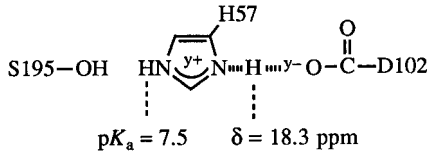
collection for a sample on the downside of the titration curve of the complex with *N*-Ac**LF**-$CF_3$ was repeated overnight and gave the same result. Furthermore, upon ionization of the H57−D102 diad in both complexes, the 15 ppm signal corresponding to the diad in free chymotrypsin appears and persists at the highest pHs. The integrity of this signal shows that the active site remains intact in these experiments. Therefore, the data points on the downsides of the titration curves for both complexes represent proton dissociation equilibria and not artifacts attributable to irreversible denaturation.

The most obvious rationale for a higher order proton release is that the dissociation of the proton from $N^{\epsilon 2}$ of the H57−D102 diad above pH 10 is accompanied by dissociation of the TFK. Hydration of the free TFK would take place quickly, and this would lead to the release of a second proton from the hydrate, the p$K_a$ of which is 9.5 (Imperiali & Abeles, 1986; Liang & Abeles, 1987).

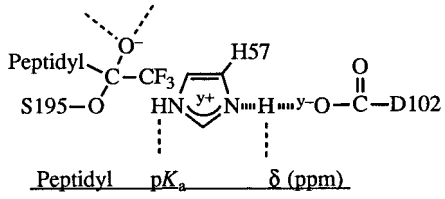
The data in Figure 3 do not exclude the possibility that a third proton may be released from the chymotrypsin−TFK complexes at higher pHs upon ionization of the diad. However, such a process would not correspond to the ionization of the N-terminal isoleucine 16. Although the p$K_a$ of this residue is 8 in free chymotrypsin, it remains protonated whenever the specificity pocket is occupied (Himoe et al., 1967; Fersht & Requena, 1971), as it is in the tetrahedral complexes with *N*-Ac**LF**-$CF_3$ and *N*-Ac**F**-$CF_3$, even at pH values above 8. The dissociation of TFKs accompanying ionization of the diad would have two consequences, the exposure of isoleucine 16 to ionization and the generation of the 3-oxyanionic form of Ser 195. Because the p$K_a$ of isoleucine 16 in free chymotrypsin is 8 and that of Ser 195 is about 14, protons released by the ionization of isoleucine 16 at pHs between 9 and 13 are taken up by Ser 195 so that the dissociation of a TFK does not lead, in and of itself, to a change in the proton content of chymotrypsin at high pHs, nor does it do so at low pHs, where the addition of Ser 195 to a TFK is also isoprotonic. If the release of a third proton should prove in the future to be associated with the steep titration curves in Figure 3, then it should arise from the ionization of another residue in chymotrypsin. The ionization of such a residue would be controlled by the protonation state of the diad.

## DISCUSSION

Structures **1** and **2** represent the active site residues of chymotrypsin in two of its chemical forms: the free enzyme in which the H57−D102 diad is protonated **1**, and peptidyl adducts formed between Ser 195 and TFKs in which the diad is protonated **2**. Included with **1** and **2** are the relevant chemical shifts for the LBHBs and information about the p$K_a$ values for the H57−D102 diad. The chemical shifts and p$K_a$ for free chymotrypsin **1** have been widely reported in the literature (Robillard & Shulman, 1972; Markley, 1978; Bachovchin, 1985). A value of 18.7 ppm for the chemical shift of complex **2**, in which the TFK is *N*-Ac**LF**-$CF_3$, has been published together with an estimate that its p$K_a$ is higher than 10 (Liang & Abeles, 1987). We here report an updated value of the chemical shift for this complex (18.9 ppm) and a more accurate value for its p$K_a$ (12.0). In addition, we report the proton chemical shift of the LBHB in the complex with *N*-Ac**F**-$CF_3$ (18.6 ppm) and the lower p$K_a$ value (10.8) for the protonated diad.




**1**




| Peptidyl | p$K_a$ | δ (ppm) |
|---|---|---|
| *N*-Ac-**LF** | 12.0 | 18.9 |
| *N*-Ac-**F** | 10.8 | 18.6 |

**2**

*Role of the LBHB in Chymotrypsin.* Tetrahedral intermediates are difficult to observe for peptide substrates of serine proteases, and substrates with very good leaving groups react through tetrahedron-like transition states rather than intermediates. Therefore, studies of the properties of tetrahedral analogs of the intermediates or transition states have been pursued to characterize their interactions with the enzyme. Among the known tetrahedral adducts of Ser 195, those formed with TFKs are more comparable in structure to the metastable intermediate than the others.[2] The present work concerns the spectroscopic and chemical characterization of the LBHBs in the adducts of chymotrypsin with *N*-Ac**LF**-$CF_3$ and *N*-Ac**F**-$CF_3$.

Occupation of the $S_2$ binding site of chymotrypsin by leucine from *N*-Ac**LF**-$CF_3$ in complex **2** gives a stronger LBHB than that formed with *N*-Ac**F**-$CF_3$, as indicated by the chemical shifts of the LBHB protons and the p$K_a$s of the diads. These parameters are correlated with the relative inhibitory efficiencies of *N*-Ac**LF**-$CF_3$ ($K_i$ = 1.2 $\mu$M) and *N*-Ac**F**-$CF_3$ ($K_i$ = 40 $\mu$M) (Brady & Abeles, 1990). The binding energy to remote amino acid residues in the inhibition of chymotrypsin by TFKs is also correlated with the substrate reactivities of analogous substrates bearing the same peptidyl groups (Brady & Abeles, 1990). The present results show that the binding of non-reacting peptidyl groups to the $S_2$ site influences the strength of the LBHB in the same way that it influences the binding of inhibitors and the reactivities of substrates, which suggests an important role for the LBHB in minimizing the activation energy.[3] The most obvious role would be to stabilize the tetrahedral

[2] Other analogs are DIP-Cht, MIP-Cht, phenylsulfonyl-Cht, and peptidylboronyl-Cht. The limitations of these as structural analogs of tetrahedral intermediates include the absence of anionic oxygen in the DIP, phenylsulfonyl, and peptidylboronyl groups; the delocalization of negative charge over two oxygens in the MIP group; the localization of a negative charge on boron in the peptidylboronyl groups; and the absence of substituents to occupy the $S_1$ and $S_2$ specificity binding sites of Cht in DIP and MIP. Peptide aldehydes form tetrahedral adducts with Ser 195, but the hemiacetal oxyanion does not occupy the oxyanion binding site. It is instead rotated into the leaving group position and forms an intimate ion pair with $N^{\epsilon 2}$ of His 57 (James et al., 1980; Delbaere & Brayer, 1985; Kurinov & Harrison, 1996).

[3] Preliminary data from one experiment on the complex of chymotrypsin with *N*-Ac**VF**-$CF_3$ indicate a p$K_a$ of approximately 12 for the H57−D102 diad based on observation of the LBHB proton, which appears at 18.8 ppm. The LBHB formed by *N*-Ac**VF**-$CF_3$ appears similar to that from *N*-Ac**LF**-$CF_3$, in conformance with inhibitory potency and the reactivity of the corresponding substrate (Brady & Abeles, 1990).

intermediate, the structure of which is likely to resemble that of the transition state. Enzyme−substrate binding interactions to all parts of the substrate in the Michaelis complex and tetrahedral intermediate, including the developing oxyanion and the remote portions of the $P_1$ to $P_4$ aminoacyl residues, as well as the incipient covalent bond to Ser 195, are likely to be important for minimizing the transition state energy.

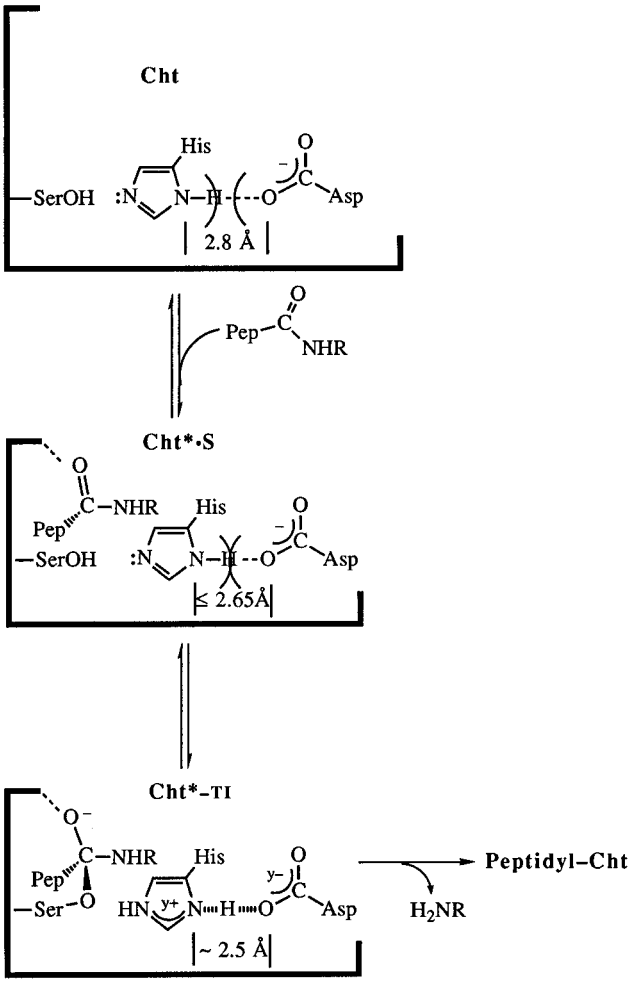
The p$K_a$ of 12 for the H57−D102 diad in the TFK complex of chymotrypsin with *N*-Ac**LF**-$CF_3$ is 5.2-log units higher than 6.8, the imidazolium p$K_a$ for glycylhistidine (Martin & Edsall, 1960) and for α-aspartylhistidine (Greenstein & Klemperer, 1939). This corresponds to 7 kcal mol$^{-1}$ of additional stabilization free energy at 5 °C for the protonated diad in the tetrahedral complex relative to the imidazolium group of histidine in peptides dissolved in water. Stabilization of the protonated diad in complex **2** is complemented by the p$K_a$ difference between 9.1 for the methylhemiketal of *N*-Ac**LF**-$CF_3$ and that of its complex with chymotrypsin, which is <4.0 (Brady et al., 1989). Therefore, the hemiketal oxyanion is also stabilized by >7 kcal mol$^{-1}$ in the chymotrpsin complex. In neutral, aqueous solution, the H57−D102 diad would be unprotonated and the hemiketal would be protonated, whereas in the enzyme complex the diad is protonated and the hemiketal is anionic. Something in the structure of enzyme-TFK complexes reverses the protonation states of these groups. Because the ionic state of the tetrahedral intermediate is ideally constituted to eliminate a leaving group, with general acid catalysis by the protonated diad, the explanation of its charge state will resolve the question of the function of the H57−D102 system in peptide bond cleavage by chymotrypsin.

According to the conventional rationale, the charge-separated state of a tetrahedral chymotrypsin complex is stabilized by ordinary electrostatic and weak hydrogen-bonded interactions. His 57 is postulated to form a conventional ion-pair with Asp 102. The positively charged imidazolium group of His 57 is further assumed to contribute long range electrostatic stabilization to the oxyanion of the tetrahedral adduct, which is further stabilized by main chain hydrogen bonds from glycine 193 and Ser 195 (Henderson, 1970; Robertus et al., 1972; Kraut, 1977; Brady et al., 1989). This interpretation does not take account of two physical properties of complexes such as **2**: (1) In a conventional ion pair, the proton bridging an imidazolium ion and a carboxylate group exhibits a proton chemical shift of <17 ppm, as in the case of 1-methylimidazolium trifluoroacetate dissolved in $CDCl_3$ (Tobin et al., 1995); however, the chemical shift of this proton in complex **2** is 18.9 ppm with *N*-Ac**LF**-$CF_3$ and 18.6 ppm with *N*-Ac**F**-$CF_3$. (2) In a conventional ion-paired ammonium carboxylate, the hydrogen bonded N−O distance is 2.80−2.90 Å, as in the crystal structures of ammonium trifluoroacetate, ammonium acetate, and ammonium hydrogen dichloroacetate (Cruickshank et al., 1964; Nahringbauer, 1967; Ichikawa, 1972). These are 0.15−0.25 Å longer than the Van der Waals contact distance of 2.65 Å between N and O. However, the distance H57−$N^{\delta1}$···H···$O^{\delta1}$−D102 in complex **2** is 2.52 Å with *N*-Ac**LF** as the peptidyl group, which is 0.13 Å shorter than the Van der Waals contact distance, and that with *N*-Ac**F** is 2.59 Å (Brady et al., 1990). Owing to the uncertainties of ±0.1 Å in the best protein crystal structures, the short N−O separation in protein crystal structural models for two hemiketal complexes of TFKs cannot adequately document an LBHB. However, conventional distances of 2.80−2.90 Å would rule out an LBHB. Inasmuch as conventional distances are not found in these complexes, the crystal structures support the assignment of an LBHB. Furthermore, all other available high-resolution crystal structures of serine proteases complexed with ketonic inhibitors as tetrahedral adducts with Ser 195 show less than Van der Waals separations for H57−$N^{\delta1}$···H···$O^{\delta1}$−D102 (Yennawar et al., 1994; Rehse et al., 1995). Moreover, high resolution refined crystal structures of free serine proteases at pH <5, in which the diad is protonated as in tetrahedral adducts, also exhibit less than Van der Waals separations for H57−$N^{\delta1}$···H···$O^{\delta1}$−D102 (James et al., 1980; Delbaere & Brayer, 1985). These distances are not compatible with simple, ion pairing between His 57 and Asp 102, but they are congruous with LBHBs and in agreement with the $^1$H NMR evidence (Frey et al., 1994).[4]

*Mechanisic Role of the LBHB.* Structural studies of serine proteases and their complexes with substrate analogs have revealed the most probable binding interactions with the tetrahedral intermediate (Blow, 1976; Henderson, 1970; Robertus et al., 1972; Stroud et al., 1974; James et al., 1980; Kossiakoff & Spencer, 1981; Delbaere & Brayer, 1985). These include subsites $S_1$to $S_4$ for binding residues $P_1$ to $P_4$ of the peptidyl moiety and main chain hydrogen bonds from glycine 193 and Ser 195 to the oxyanion. The binding interactions have been interpreted to induce a distortion of the scissile carbonyl group toward pyramidal geometry accompanied by a weakening of the bond to the leaving group and an increase in the electrophilic reactivity of the carbonyl carbon (Kraut, 1977; James et al., 1980). High-resolution structures of pancreatic and soy bean trypsin inhibitor complexes with trypsin support the pyramidal distortion of the carbonyl group (Bode et al, 1976). Alternative interpretations have raised questions about the degree of distortion (Steitz & Shulman, 1982). The apparent fact that His 57 (p$K_a$ = 7.5) could not be an effective general base in abstracting a proton from Ser 195 (p$K_a$ = 14) has inspired mechanistic postulates in which the reaction of Ser 195 with the peptide acyl carbonyl group takes place spontaneously (Kraut, 1977; James et al., 1980). The role of the diad H57−D102, which is clearly important (Craik et al., 1987), is not specified by these mechanisms, although His 57 has been thought to guide the migration of the proton from the serine hydroxyl group to the leaving group of the tetrahedral intermediate (Kraut, 1977), perhaps after the formation of the intermediate. We here propose an extension of this theory that retains its essential conformational, hydrogen bonding, and electrostatic components but differs in postulating that His 57 actually serves as the general base−general acid catalyst for the formation and breakdown of the tetrahedral intermediate.

[4] J. L. Markley and M. Westler measured the fractionation factor for the low-field proton in the protonated triad of chymotrypsinogen and found it to be 0.4 (Markley & Westler, 1996). They also found $\Delta G^{\ddagger}$ and $\Delta H^{\ddagger}$ for the exchange of the low-barrier hydrogen bonded proton with protons of solvent to be 12.4 and 10.7 kcal/mol, respectively. Because the fractionation factor and activation energy for exchange of the bridging proton in the deprotonated diad were found to be normal, they concluded that the proton in the LBHB was more strongly hydrogen bonded than in the unprotonated diad, in agreement with the earlier postulate (Frey et al., 1994). C. J. Halkides et al. (1996) have measured the fractionation factor for the low-field proton in the protonated triad of subtilisin and found it to be 0.6. These fractionation factors are compatible with LBHBs. The authors are grateful to these colleagues for communicating their results prior to publication.

Scheme 2

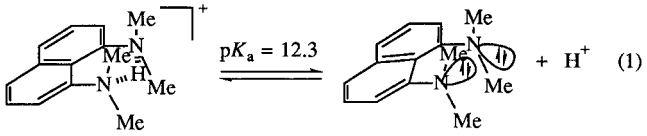



Consider the mechanism in Scheme 2. The initial binding of a specific substrate to the free enzyme (**Cht**) drives a conformational change to form the Michaelis complex (**Cht***S*), in which the substrate carbonyl group is twisted into a pyramidal conformation and steric compression is introduced between His 57 and Asp 102 of the enzyme. In principle, compression in the diad might be relieved through LBHB formation; however, this cannot take place in the Michaelis complex because of the gross mismatch between the p$K_a$s of the Asp 102 $\beta$-carboxyl group and the *unprotonated* His 57 imidazole group. The p$K_a$s of the $\beta$-carboxyl group and the imidazolium group of *protonated* His 57 can be matched, however, and in the protonated diad LBHB formation would relieve the strain between His 57 and Asp 102. Steric compression thereby becomes the means through which proton transfer is facilitated from Ser 195 to $N^{\epsilon 2}$ of His 57; LBHB formation allows relief of strain between His 57 and Asp 102. The basicity of His 57 in the diad is increased by this mechanism, thereby facilitating the deprotonation of Ser 195 in concert with nucleophilic attack on the substrate acyl carbonyl group at neutral pHs. It has previously been postulated that the p$K_a$ of His 57 increases in the course of the reaction of substrates bound to the active site of chymotrypsin (Liang & Abeles, 1987; Finucane & Malthouse, 1992). Such an increase in basicity is explained by the mechanism of Scheme 2. The conformational change in Scheme 2 is not envisioned as a major or global change in the structure of chymotrypsin. A conformational transition that brings about a compression of a few tenths of an Angstrom in the diad would be in accord with published inhibitor-induced changes in serine proteases (James et al., 1980), and this would be sufficient to induce protonation of the diad and LBHB formation.

The following lines of evidence support Scheme 2: (a) Tetrahedral complexes of TFKs with chymotrypsin (complexes **2**) display high p$K_a$s. (b) These complexes incorporate LBHBs between His 57 and Asp 102, as documented by low-field chemical shifts and close contacts in violation of Van der Waals contact distances. (c) A substrate-induced conformational transition such as that in Scheme 2 has been documented for serine proteases by the structural analyses of enzyme−inhibitor complexes (Kraut, 1977; James et al., 1980). (d) Steric crowding and close contacts in the active site of the Michaelis complex, a prerequisite for LBHB formation in this mechanism, are also uniformly reported for complexes of serine proteases with inhibitors (Kraut, 1977; James et al., 1980; Steitz & Shulman, 1982). (e) The close proximity of His 57-C-2(H) to a main chain carbonyl group in all known structures of serine proteases further documents the steric crowding in this site (Derewenda et al., 1994). All currently known structural and spectroscopic properties of serine proteases either support or are compatible with the mechanism in Scheme 2.

A novel aspect of the mechanism in Scheme 2 is that relief of strain between $N^{\delta 1}$ of His 57 and Asp 102 manifests itself *transannularly* as increased basicity at $N^{\epsilon 2}$ of His 57. This allows increased basicity to be brought to bear near the 3-hydroxyl group of Ser 195, remote from the locus of highest steric compression. The imidazole ring of histidine is supremely suited to this function.

Increased basicity through relief of strain in the diad is analogous to the phenomenon by which proton sponge molecules display high basicity (eq 1). Electronic repulsion




(1)

is enforced in *N*,*N*,*N*′,*N*′-tetramethyl-1,8-diaminonaphthalene on the right side of eq 1 by the steric requirements of the four methyl groups. This repulsion manifests itself as electronic repulsion between the nonbonding electron pairs on N and N′, which leads to steric distortion of the fused aromatic rings (Einspahr et al., 1973). Protonation to form an LBHB on the left side of eq 1 relieves the strain and allows the rings to become coplanar (Coult et al., 1992). Relief of strain upon protonation imparts high basicity to the nitrogens and is one of the factors that account for the p$K_a$ of 12.3 compared with 4.6 for the parent 1,8-diaminonaphthalene (Hibbert & Emsley, 1990). Steric inhibition of resonance could account for a p$K_a$ of about 9 but not 12.3. The p$K_a$ is increased to 16.1 by the additional presence of methoxyl substituents in the 3 and 7 positions. The function of the LBHB in Scheme 2 is analogous, except that the strain is introduced through binding interactions between the substrate and the enzyme, whereas the strain in the proton sponges is introduced through organic synthesis.

*p$K_a$ of the H57−D102 Diad in Free Chymotrypsin.* The p$K_a$ at $N^{\epsilon 2}$ of the diad H57−D102 in free chymotrypsin has been measured by NMR spectroscopy and reported to be 7.5 at 3 °C when monitored by the transition of the low-field proton (Robillard & Shulman, 1974) or 6.2 at 31 °C when monitored by the transition of the C-2 imidazole proton

(Markley & Ibañez, 1978). The p$K_a$ value of 7.5 for free chymotrypsin will be adopted for the present discussion because the present work was carried out at 5 °C with observation of the low-field proton.

The existence of LBHBs in the protonated diads of tetrahedral complexes and free chymotrypsin does not explain the differences in the diad p$K_a$s between the free enzyme **1** on one hand and TFK complexes **2** on the other. Scheme 2 suggests the basis for the normal p$K_a$ of the diad in free chymotrypsin despite the presence of an LBHB. In a TFK complex **2** and the tetrahedral adduct in Scheme 1, the energy required for the protein conformational change is provided by binding energy from the peptidyl group and the oxyanion, and the diad exhibits a high p$K_a$. This can be described in terms of free energy by eqs 2−4 for tetrahedral adduct formation, and eqs 2 and 5 for the protonation of free chymotrypsin.

$$\mathbf{Cht} = \mathbf{Cht^*} \qquad \Delta G_2^\circ > 0 \qquad (2)$$

$$\mathbf{Cht^*} + \mathrm{S} = \mathbf{Cht^*}\mathrm{S^*} \qquad \Delta G_3^\circ \ll 0 \qquad (3)$$

$$\mathbf{Cht^*}\mathrm{S^*} = \mathbf{Cht^*} - \mathrm{TI} \qquad \Delta G_4^\circ \approx 0 \qquad (4)$$

In the formation of the tetrahedral intermediate (TI), the free energy required for the conformational change in eq 2 is positive, and that for binding the substrate to the conformationally altered enzyme in eq 3 is very negative. The free energy for the formation of the Michaelis complex is the sum $\Delta G_2^\circ + \Delta G_3^\circ < 0$; that is, the binding free energy is negative overall, but the measured binding energy is for the sum of eqs 2 and 3 and is less negative than that for eq 3. The TI complex contains an LBHB in the diad, which exhibits its characteristic high p$K_a$.

Protonation of the diad in free chymotrypsin also requires a change in conformation to one that allows LBHB formation, but substrate binding energy is not available. Therefore, the energy for the conformation change is supplied by the LBHB itself, as described by eq 2 (above) and eqs 5 and 6.

$$\mathbf{Cht^*} + \mathrm{H^+} = \mathbf{Cht^*}{-}\mathrm{H^+} \qquad \Delta G_5^\circ \ll 0 \qquad (5)$$

$$\mathbf{Cht} + \mathrm{H^+} = \mathbf{Cht^*}{-}\mathrm{H^+} \qquad \Delta G_6^\circ = \Delta G_2^\circ + \Delta G_5^\circ \qquad (6)$$

Because proton binding is coupled to the conformational change, the apparent affinity of the diad for protonation in the free enzyme is correspondingly less than that for the conformationally altered enzyme in the TFK complexes **2** and presumably also in the tetrahedral intermediate. This mechanism is an example of the use of binding energy to remote portions of a substrate to catalyze an enzymatic reaction (Jencks, 1975).

The mechanism in Scheme 2 presents a clear and attractive rationale for the means by which chymotrypsin can increase the reactivity of Ser 195 as a nucleophile by making use of the LBHB to increase the basicity of His 57. The substrate-induced conformational change lends practical value to the LBHB as a means to increase the basicity of the diad. Were it not for the conformational change, the diad would exhibit a high p$K_a$ in free chymotrypsin and be fully protonated in neutral solutions, thereby disabling His 57 as a base. The mechanism in Scheme 2 cures this potential problem by allowing the diad p$K_a$ to be high only when a substrate is bound and the addition of Ser 195 to the carbonyl group is imminent or in progress. This postulate explains general base function of His 57 on the basis of low-barrier hydrogen bonding in the tetrahedral intermediate (Frey et al., 1994). To the extent that the LBHB is formed in the transition state, the rate of tetrahedral adduct formation is increased. In the reactions of substrates that have very good leaving groups, such as *p*-nitrophenyl esters, which do not require protonation, no discrete tetrahedral intermediates are formed and the reactions are concerted.[5] Steric compression in the diad can still increase the basicity of $N^{\epsilon 2}$ and the reactivity of Ser 195. In this case, strong hydrogen bonding between His 57 and Asp 102 stabilizes the transition state.

*Rate Enhancement Brought about by the LBHB.* On the basis of the mechanism in Scheme 2, it would be difficult to assign the rate enhancement factor specifically provided by the LBHB because the formation of this hydrogen bond depends on many other interactions, the failure of any one of which would prevent LBHB formation. It has been pointed out that any structural perturbation in the H57−D102 diad that disrupts the LBHB causes a decrease in acylation rate by a factor of $10^4$−$10^5$ (Frey et al., 1994). This corresponds to an increase in activation energy of 5−7 kcal mol$^{-1}$, a value that is remarkably similar to the difference in $\Delta G^\circ$ ($\geq$7 kcal mol$^{-1}$) for the LBHB in the TFK complex **2** compared with the conventionally hydrogen bonded imidazolium ion. Therefore, the contribution of the LBHB to catalysis is very important but not the only factor in catalysis. All of the interactions between the substrate and the enzymatic binding site contribute to decreasing the entropy of the reacting functional groups and to bringing them into close proximity in the correct orientations. We propose that the LBHBs in serine proteases increase the chemical reactivity of Ser 195 by increasing the basicity of His 57.[6] The most effective acid−base catalyst for the mechanism of peptide cleavage in the acylation of chymotrypsin would be one that is strong enough as a general base to abstract a proton from Ser 195 but not so strong that its conjugate acid form would be unable to protonate the leaving group. Such a general base would display a p$K_a$ value that is intermediate between that of the leaving amino group (8−9) and that of Ser 195 (≈14). The values of p$K_a$ = 10.7

[5] Private communication from R. A. Hess, A. C. Hengge, and W. W. Cleland on isotope effects in the enzymatic hydrolysis of *p*-nitrophenyl esters.

[6] In the conventional rationale for the stabilization of tetrahedral intermediates in chymotrypsin and analogous serine proteases, the main chain hydrogen bonds from glycine 193 and Ser 195 stabilize the oxyanion, and the resulting electrostatic interaction with His 57 keeps it protonated (Henderson, 1970; Kraut, 1977; Robertus et al., 1972; James et al, 1980). This might account for the fact that the p$K_a$ of His 57 in DIP chymotrypsin is 7.4, whereas that of MIP chymotrypsin, the partially hydrolyzed and monoanionic form, is 10.3 (Adebodun & Jordan, 1989). On this basis, the sum of oxyanion binding and electrostatic interaction with His 57 can account for 4 kcal mol$^{-1}$ of stabilization at 25 °C. The relevance of MIP chymotrypsin to the tetrahedral adduct is limited, however, by the fact that the negative charge in the MIP group is delocalized between two oxygens, one of which is in the oxyanion binding site and the second is intimately ion paired with His 57 (Kossiakoff & Spencer, 1981). Because the oxyanion in a tetrahedral intermediate or TFK adduct is localized in its binding site, and is not ion-paired with His 57, the electrostatic stabilization will be much smaller than in MIP chymotrypsin.

and 12 for His 57 in chymotrypsin TFK adducts (Figure 3) lie within this range.

*Strength of the LBHB.* The strengths of covalent bonds in the range 50−120 kcal mol$^{-1}$ have generally been expressed in enthalpic terms because of the minor entropic contributions to large bond energies. Entropic contributions can be significant in the strength of hydrogen bonds, which are weaker than conventional covalent bonds, and we here consider hydrogen bond strength in free energy terms. We have provided evidence that the LBHB in the TFK complex of chymotrypsin with *N*-Ac**L**-**F**-$CF_3$ is 7 kcal mol$^{-1}$ stronger than a conventional imidazolium hydrogen bond. A typical conventional hydrogen bond is thought to provide 3−5 kcal mol$^{-1}$ of stabilization. If we accept 4 kcal mol$^{-1}$ as a conservative value for an imidazolium hydrogen bond to water, the strength of the LBHB in the *N*-Ac**L**-**F**-$CF_3$ complex of chymotrypsin would be 11 kcal mol$^{-1}$. This refers to the relative energies of two states, one in which the LBHB is intact and one in which the structure is identical except for the absence of a hydrogen bond.

*Importance of the LBHB for Binding TFKs.* According to the rationale presented here for the NMR spectra and titration curves, ionization of the chymotrypsin adduct of *N*-Ac**L**-**F**-$CF_3$ follows the course of eqs 7−12, where $K_a^{57}$, $K_a^{195}$, and $K_a^{16}$ are dissociation constants.

$$\begin{matrix}I_{16}\text{–}H^+\\ S_{195}\text{–}O\text{–TFK}\\ H_{57}\text{–}H^+\end{matrix} \xrightleftharpoons{K_a^{57}} \begin{matrix}I_{16}\text{–}H^+\\ S_{195}\text{–}O\text{–TFK}\\ H_{57}\end{matrix} + H^+ \qquad (7)$$

$$\begin{matrix}I_{16}\text{–}H^+\\ S_{195}\text{–}O\text{–TFK}\\ H_{57}\end{matrix} \xrightleftharpoons{K_d} \begin{matrix}I_{16}\text{–}H^+\\ S_{195}\text{–}O^-\\ H_{57}\end{matrix} + \text{TFK=O} \qquad (8)$$

$$\begin{matrix}I_{16}\text{–}H^+\\ S_{195}\text{–}O^-\\ H_{57}\end{matrix} + H^+ \xrightleftharpoons{1/K_a^{195}} \begin{matrix}I_{16}\text{–}H^+\\ S_{195}\text{–}OH\\ H_{57}\end{matrix} \qquad (9)$$

$$\begin{matrix}I_{16}\text{–}H^+\\ S_{195}\text{–}OH\\ H_{57}\end{matrix} \xrightleftharpoons{K_a^{16}} \begin{matrix}I_{16}\\ S_{195}\text{–}OH\\ H_{57}\end{matrix} + H^+ \qquad (10)$$

$$\text{TFK=O} + H_2O \xrightleftharpoons{K_h} \text{TFK(OH)}_2 \qquad (11)$$

$$\text{TFK(OH)}_2 \xrightleftharpoons{K_{ha}} \text{TFK(OH)–O}^- + H^+ \qquad (12)$$

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$$\begin{matrix}I_{16}\text{–}H^+\\ S_{195}\text{–}O\text{–TFK}\\ H_{57}\text{–}H^+\end{matrix} \xrightleftharpoons{K_a} \begin{matrix}I_{16}\\ S_{195}\text{–}OH\\ H_{57}\end{matrix} + \text{TFK(OH)–O}^- + 2H^+ \qquad (13)$$

The overall process is described by eq 13 above. The equilibrium constant $K_a$ for eq 13 is given by the product of the equilibrium constants for eqs 7−12 (eq 14) and corresponds to the acid dissociation constants evaluated from the titration curves in Figure 3. It would be of interest to know the value of $K_d$, the equilibrium constant for the dissociation of a TFK from chymotrypsin according to eq 8, in which His 57 is not protonated. This value compared with that for dissociation of the same TFK when His 57 is protonated would indicate the degree to which the protonated diad stabilizes the TFK adduct. Although values for most of the constants on the right side of eq 14 are available in the literature, they have been obtained at ambient temperatures, whereas the values of $K_a$ reported here were obtained at 5 °C in order to obtain good resolution in the NMR spectra. It is useful to consider what can be revealed by eq 14. The value of $K_a^{16}$ is $10^{-8}$ M (Himoe et al., 1967; Fersht & Requena, 1971). No measured value for $K_a^{195}$ is available, although $10^{-14}$ M is a reasonable estimate based on the p$K_a$ values for related alcohols (Jencks & Regenstein, 1970). Values for other constants are available for the case of *N*-Ac**L**-**F**-$CF_3$ and are likely to be similar for *N*-Ac**F**-$CF_3$: $K_h = 4.5 \times 10^3$ (Brady & Abeles, 1990) and $K_{ha} = 3 \times 10^{-10}$ (Imperiali & Abeles, 1986; Liang & Abeles, 1987). Substitution of these into eq 14 gives eq 15, which relates the ratio of $K_a/K_a^{57}$ to $K_d$, where $C = (1/K_a^{195})(K_a^{16})(K_h)(K_{ha})$.

$$K_a/K_a^{57} = CK_d \qquad (15)$$

From Figure 3 and the fact that the dissociation of the proton from $N^{\epsilon 2}$ of the H57−D102 diad is the defining event in the dissociation of *N*-Ac**L**-**F**-$CF_3$ according to eq 8, it may be concluded that $K_a^{57} \leq K_a = 10^{-12}$ M; that is, the value of p$K_a^{57}$ for $N^{\epsilon 2}$ in the chymotrypsin TFK adduct must be at least 12, although it may be larger depending on the value of $K_d$. If $K_a^{57}$ and $K_a$ are equal ($10^{-12}$ M from Figure 3), the value of $K_d$ for *N*-Ac**L**-**F**-$CF_3$ will be $1/C$. We cannot calculate $1/C$ at 5 °C; however, it would be 0.7 M if the parameters that define $C$ were the same at 5 °C as at ambient temperatures. In any reasonable scenario, $K_d$ is likely to be immeasurably large, as is indicated by the NMR spectra. Therefore, the protonated diad and its associated LBHB are crucially important to the binding of *N*-Ac**L**-**F**-$CF_3$. If $K_a^{57}$ is smaller than $K_a$, then $K_d$ will be even larger, and the value of p$K_a^{57}$ will be higher than 12.

The fact that the ionization of $N^{\epsilon 2}$ in the H57−D102 diad is coupled to dissociation of the chymotrypsin TFK adduct introduces a formal ambiguity in the interpretation of the differences in p$K_a$ values for the adducts formed with *N*-Ac**L**-**F**-$CF_3$ and *N*-Ac**F**-$CF_3$. Because eq 15 shows that p$K_a^{57}$ depends on both $K_a$ and $K_d$, it is conceivable that the difference between the values of $K_a$ shown by Figure 3 could be due entirely to a difference in $K_d$ for the two molecules rather than to a difference in $K_a^{57}$. If this is true, then the value of p$K_a^{57}$ must be equal to or higher than 12 for complexes with both *N*-Ac**L**-**F**-$CF_3$ and *N*-Ac**F**-$CF_3$. Moreover, the value of $K_d$ for *N*-Ac**F**-$CF_3$ would be 40 times larger than that for *N*-Ac**L**-**F**-$CF_3$. In either case, the binding of TFKs in the reversal of eq 8 is indeterminably weak. And, in either case, the main conclusion from this work remains, namely that the value of p$K_a^{57}$ for the adducts is very high, at least 12 for that formed with *N*-Ac**L**-**F**-$CF_3$, and this represents the stabilization brought about by the LBHB.

*Complexes of Chymotrypsin with Naturally Occurring Protease Inhibitors.* Biophysical data on complexes of chymotrypsin and trypsin with ovomucoid, BPTI, and SBTI are also compatible with the mechanism in Scheme 2. A high-resolution X-ray crystal structure of the complex formed between chymotrypsin and ovomucoid shows a separation between $N^{\delta 1}$ of His 57 and $O^{\delta}$ of Asp 102 of 2.81Å, which is too long to allow for LBHB formation (Fujinaga et al., 1987). NMR data on the C-2(H) and $N^{\delta 1}$ protons of His 57

in complexes of chymotrypsin and trypsin with SBTI or BPTI show that His 57 is positively charged; however, the low-field protons are too far upfield (14.6 and 13.3 ppm, respectively) to be engaged in LBHBs (Markley, 1978; Markely & Ibañez, 1978). These results show that LBHB formation in complexes of chymotrypsin and trypsin with these inhibitors is compromised. The proteinase inhibitors presumably stabilize conformations of chymotrypsin and trypsin that do not allow sufficiently close approach of His 57 and Asp 102 to permit LBHB formation. Peptide cleavage in these inhibited complexes would be very slow, according to the mechanism in Scheme 2, because the conformational transition to Cht* would be blocked by the inhibitors. Release of the cleaved peptides is also very slow in these complexes.

*Significance of LBHBs for Proteases.* The LBHBs in the protonated diads of serine proteases are postulated to enhance the reactivity of Ser 195 by increasing the basicity of His 57. The reactivity of Ser 195 as a nucleophile is a particular problem in the cleavage of the peptide amide linkage, which is highly resistant to nucleophilic attack. Esterases and $\beta$-lactamases generally incorporate serine residues as catalytic groups, but they do not include counterparts to the H57−D102 diad or involve an LBHB. Esters and $\beta$-lactams are far more chemically reactive than peptide amides, and LBHBs presumably do not participate in the hydrolysis of these more reactive molecules by enzymes other than serine proteases. In cysteine proteases, the problem of nucleophilic reactivity does not arise, because the active site cysteine exists as the highly nucleophilic thiolate ion, stabilized as such by the imidazolium ring of a histidine residue. These enzymes do not contain a counterpart to Asp 102, which is either absent or replaced by an Asn residue. Therefore, cysteine proteases presumably do not make use of an LBHB in the manner of the serine proteases. It is not known whether LBHBs participate in the action of aspartic proteases.

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