A Low-Barrier Hydrogen Bond in Subtilisin: ^{1}H and ^{15}N NMR Studies with Peptidyl Trifluoromethyl Ketones†

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ABSTRACT: The Nδ1 proton of His 64 forms a hydrogen bond with Asp 32, as part of the catalytic triad in serine proteases of the subtilisin family. His 64 in subtilisin has been studied by ^{1}H and ^{15}N NMR spectroscopy in the presence and absence of peptidyl trifluoromethyl ketones (TFMKs) that are transition state analog inhibitors. For subtilisin Carlsberg, the downfield resonance of the imidazolium Nδ1 proton is approximately 18.3 ppm and the D/H fractionation factor is 0.55 ± 0.04 at pH 5.5 (11 °C), and 0.63 ± 0.04 (5 °C) and 0.68 ± 0.04 at pH 6 (11 °C). In the complex between subtilisin Carlsberg and Z-L-leucyl-L-leucyl-L-phenylalanyltrifluoromethyl ketone (Z-LLF-CF_3) at pH values between 6.5 and 10.6, His 64 remains positively charged, and the D/H fractionation factor of its Nδ1 proton is 0.85 ± 0.05. In the complex between a subtilisin variant from *Bacillus lentus* and Z-LLF-CF_3, the proton resonance at 18.8 ppm is correlated with a ^{15}N resonance at 197.6 ppm downfield from liquid NH_3 with a $^1J_{NH}$ of 81 Hz. The chemical shifts of subtilisin complexes with peptidyl TFMKs are among the most downfield shifts reported for any protein. At pH 9.5, His 64 is neutral and the D/H fractionation factor increases to 1.2 with a chemical shift of 15.0. His 64 is positively charged in the free enzyme at low pH, the inhibitor hemiketal complex at neutral pH, and the transition state for amide bond hydrolysis. These data thus provide indirect evidence for the presence of a low-barrier hydrogen bond in the catalytic mechanism of subtilisin proteases.

Several workers have considered the possibility that short, strong hydrogen bonds stabilize the transition states of enzymes in general (Cleland, 1992; Gerlt & Gassman, 1993; Cleland & Kreevoy, 1994; Zhao et al., 1996) and serine proteases in particular (Schowen, 1988; Frey et al., 1994). One of the properties of these low-barrier hydrogen bonds (LBHBs)[1] is that the distance between donor and acceptor atoms has decreased relative to an ordinary hydrogen bond, causing the barrier against proton transfer to fall to approximately the zero-point energy of the hydrogen atom (Hibbert & Emsley, 1990). LBHBs are stronger than ordinary hydrogen bonds. However, unlike the case for single-well H bonds, the hydrogen atom is not found to be equidistant between donor and acceptor atoms (Hibbert & Emsley, 1990).

The mechanism by which serine proteases catalyze the hydrolysis of amides has received much attention from mechanistic and structural enzymologists, and the role of the aspartic acid in the catalytic triad has not been entirely clear. Recently, it has been demonstrated that a LBHB forms between the conserved histidine and aspartic acid residues within the catalytic triad of chymotrypsin, and it was suggested that this LBHB can facilitate catalysis (Frey et al., 1994).[2] The presence of a LBHB in serine proteases is supported by model chemistry showing the formation of LBHBs between ammonium ions and carboxylate ions in organic solvents (Tobin et al., 1995; Smirnov et al., 1996). Nevertheless, the presence of LBHBs at the active sites of enzymes remains controversial (Warshel et al., 1995; Shan et al., 1996). We reiterate that the LBHB proposal does not imply that the proton is found equidistant from the oxygen of the aspartate and the nitrogen of the imidazole within the catalytic triad, and we note that this proposal claims to explain only about 6 kcal/mol of activation energy, a value that is similar to a recent measurement on a LBHB in the maleate monoanion in solution (Schwartz & Drueckhammer, 1995).

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[1] Abbreviations: CAPS, 2-(cyclohexylamino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; DMSO, dimethyl sulfoxide; HMQC, heteronuclear multiple-quantum coherence; k_{cat}, enzymatic turnover number; K_m, Michaelis constant; LBHB, low-barrier hydrogen bond; MES, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; sAAPFpNA, *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-*p*-nitroanilide; TFMK, trifluoromethyl ketone; Z, *N*-(carbobenzoxy); Z-LAF-CF_3, Z-L-leucyl-L-alanyl-L-phenylalanyltrifluoromethyl ketone; Z-LLF-CF_3, Z-L-leucyl-L-leucyl-L-phenylalanyltrifluoromethyl ketone; Z-AAPF-CF_3, Z-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyltrifluoromethyl ketone; S/N, signal-to-noise ratio. The amino acid numbering corresponds to that of mature *Bacillus amyloliquefaciens* (subtilisin BPN′).

[2] This mechanism is distinct from the proposal of Schowen and coworkers (Schowen, 1988) in that it localizes the LBHB to the His/Asp dyad, among other differences.

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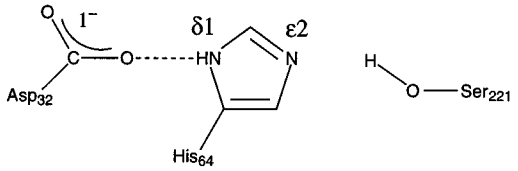
Chart 1



The bacterial serine proteases are unrelated in primary sequence or secondary structure to trypsin-like proteases yet display a similar three-dimensional arrangement of the catalytic triad (Chart 1), consisting of Ser 221, His 64, and Asp 32 in the case of subtilisin Carlsberg (Matthews et al., 1977; Lange et al., 1994). His 64 acts as a general base to deprotonate Ser 221 to form a tetrahedral intermediate. In the structure of the enzyme-bound transition state, His 64 is protonated and the tetrahedral alkoxide ion is ionized. Furthermore, both subtilisin BPN′ (Jackson & Fersht, 1993) and chymotrypsin (Brady et al., 1989) are inhibited by peptidyl TFMKs. These compounds form covalent adducts with the active site Ser 221 and are transition state analogs that resemble the tetrahedral intermediate along the reaction pathway of the enzyme (Brady & Abeles, 1990; Brady et al., 1990). A demonstration that subtilisins with widely differing sequences also possessed LBHBs between the imidazolium ion of His 64 and Asp 32 at low pH and in the presence of a transition state analog inhibitor would confirm the general importance of such H bonds in catalysis by serine proteases.

NMR provides several kinds of information which are evidence for or against the presence of a LBHB. These include the low-field chemical shift of the proton, which is typically 16−20 ppm for such bonds, as well as the ^{15}N chemical shift of the hydrogen bond donor. A third parameter is the equilibrium deuterium/protium (D/H) fractionation factor, ϕ, defined by eq 1 that is expected to be

$$AH + DOH \overset{\phi}{\rightleftharpoons} AD + HOH \qquad (1)$$

significantly less than 1 for LBHBs (Hibbert & Emsley, 1990). Because normal hydrogen bonds occasionally possess atypical fractionation factors (Hibbert & Emsley, 1990; Loh & Markley, 1994; Edison et al., 1995), it is desirable to gather more that one type of information about the hydrogen bond under study. Here, we report the unusually downfield proton and ^{15}N chemical shifts as well as D/H fractionation factors of His 64 in subtilisin under three conditions: the free enzyme at pH 5.5−6.0, the enzyme complexed with peptidyl TFMKs, and the free enzyme at pH 9.5. The former two resemble the high-energy tetrahedral intermediate in which Nϵ2 of the catalytic histidine becomes positively charged, while at pH 9.5 in the free enzyme (the ground state), Nϵ2 of the catalytic histidine is neutral.

MATERIALS AND METHODS

Sample Preparation. Subtilisin Carlsberg was purchased from Sigma (St. Louis, MO) as lyophilized protein or purified from a sample of *Bacillus licheniformis* subtilisin using bacitracin-Sepharose chromatography (Grøn et al., 1990). Subtilisin BPN′ was purchased from Boehringer Mannheim. A subtilisin variant from *Bacillus lentus* (Goddette et al., 1992) was purified and the protein concentration determined by active site titration with phenylmethanesulfonyl fluoride as previously described (Hsia et al., 1996). The enzyme was labeled with ^{15}N (99% ^{15}N) by growing cultures on Isogro powder (Isotec, Inc.).

Solutions of purified subtilisins were first dialyzed versus 1 mM KH_2PO_4 (pH 6.0) and 5 mM KCl at 6 °C, divided into equal aliquots, and lyophilized. For studies involving the free enzyme, the lyophilized enzyme was dissolved in 50 mM MES (pH 5.5 and 6.0) or 50 mM CHES (pH 9.5). In most cases, 0.05% Tween-20 and 0.02% sodium azide were present. The effects of freezing the enzyme in the presence or absence of glycerol or of lyophilization on the activity of the enzyme relative to no treatment were found to be at or below the level of experimental error.

For studies of peptidyl TFMK complexes, solutions of Z-LAF-CF_3 or Z-LLF-CF_3 (∼60 mM) were prepared in DMSO. Samples of lyophilized protein were dissolved in various mixtures of H_2O and D_2O; the final concentration of buffer was 100 mM K_2HPO_4 (pH 7.5) and 0.05% Tween-20. The final concentration of Z-LLF-CF_3 was typically 2−3 mM, the final concentration of DMSO 2−6%, and the concentration of enzyme 1.5 mM. For experiments at higher pHs, 100 mM CHES (pH 9.5) and 0.05% Tween-20 and 100 mM CAPS (pH 10.6) and 0.05% Tween-20 were prepared at 24 °C. For all NMR experiments, the pH is reported at the temperature of the NMR experiment (11 or 5 °C) and is not corrected for the mole fraction of deuterium.

Kinetics. Kinetic assays were performed at 25 °C essentially as previously described (Jackson & Fersht, 1993). The apparent kinetic parameters found for the hydrolysis of sAAPFpNA at pH 7.5 in 100 mM potassium phosphate and 0.05% Tween-20 were a K_m of 235 μM and a k_{cat} of 390 s^{-1}. At pH 8.6 in 100 mM Tris HCl and 0.005% Tween-80, K_m = 260 μM and k_{cat} = 500 s^{-1}. These values are in good agreement with previously reported values for K_m of 200 μM and for k_{cat} of 510 s^{-1} (Wells et al., 1987). The pK_a of the active site His 64 (Nϵ2H) for subtilisin Carlsberg was determined in 20 mM ethylenediamine buffers at an ionic strength of 0.05 M maintained with KCl, as previously described (Russell et al., 1987).

For inhibition studies, progress curves were measured spectrophotometrically at pH 7.5 in 100 mM potassium phosphate and 0.05% Tween-20 using procedures described previously (Brady & Abeles, 1989; Jackson & Fersht, 1993). The enzyme concentration was 0.5 nM, and the substrate sAAPFpNA was present at ca. 2K_m (530 μM). Inhibitor concentrations ranged from 0.4 to 4.4 μM for Z-LLF-CF_3 and from 2.1 to 8.5 μM for Z-LAF-CF_3. The data were fit to eq 2:

$$A = v_s t + (v_0 - v_s)[1 - \exp(-k_{obs} t)]/k_{obs} \qquad (2)$$

where A is the absorbance at 412 nm, v_s is the steady state rate after the onset of inhibition, v_0 is the steady state rate in the absence of the inhibitor, and k_{obs} is the first-order rate constant for the approach to steady state. K_i and k_{on} were calculated from progress curves at several inhibitor concentrations using eqs 3 and 4.

$$K_i = v_s[I]/(v_0 - v_s)(1 + [S]/K_m) \qquad (3)$$

$$k_{obs} = k_{off} + k_{on}[I]/(1 + [S]/K_m) \qquad (4)$$

k_{off} was too small to be measured at the concentration of inhibitors used in these experiments.

NMR Spectroscopy. Proton NMR spectra at 500 MHz were taken using a Bruker AMX-500 spectrometer or a GE GN-500 spectrometer and at 750 MHz using a Bruker DMX-750 spectrometer. Spectra of subtilisin (ca. 1.5 mM) at 500 MHz with various ratios of H_2O/D_2O were recorded at 11 °C, taking 5000−7500 scans. The spectral width was 16 129 Hz, and 4096 complex data points were collected with a 200 ms delay between scans. A jump−return sequence was used for maximum excitation of the signal of interest and minimum excitation of water. For the determination of the fractionation factor in the presence of Z-LLF-CF_3 at 500 MHz and for all experiments at 750 MHz, sodium 3-(trimethylsilyl)-1-propanesulfonate was present as an integration standard in a coaxial tube that was transferred from one sample to the next. Spectra at 750 MHz were acquired at 5 °C using either a jump−return (spectral width = 24 272 Hz; postacquisition delay = 200 ms) or an 1−1 (spectral width = 30 030 Hz; delay = 230 ms) sequence for selective excitation. The block size was 4096 complex points, and 6000 transients were collected. The enzyme was shown not to lose activity over the course of an NMR experiment (∼1 h) at pH 7.5, either in the presence or in the absence of inhibitor. Variation of the postacquisition delay at either field did not change the integrals significantly. Addition of 100 μM $CaCl_2$/5 μM $MnCl_2$ (to relax the hydrogen nuclei of water more quickly) had no effect on the integral of the downfield resonance. HMQC (forbidden echo) spectra were acquired with jump−return pulses (Roy et al., 1984). Nitrogen chemical shifts were referenced to liquid ammonia by setting the zero value of the ^{15}N frequency scale equal to 0.101 329 118 times the proton zero frequency (Wishart et al., 1995).

NMR spectra were processed by zero filling to 8K points and exponential multiplication of 5−20 Hz using FELIX (Biosym Research, San Diego, CA). Baselines were corrected with a 5−7th order polynomial. Coupling constants were found by nonlinear least-squares fitting of the signal. Integrals were determined by drawing baselines to the signal of interest and then by cutting and weighing. The data were fitted using Grafit (Erithacus Software, Ltd.) or Kaleidagraph (Synergy Software) to

$$y^{-1} = C[\phi(1 - x)/x + 1] \quad (5)$$

where y is the value of the integral, C is a normalization factor, x is the mole fraction of H_2O, $1 - x$ is the mole fraction of D_2O, and ϕ is the D/H fractionation factor. The fit was weighted using the integral to the 4th power (Loh & Markley, 1994).

RESULTS

Kinetics of Inhibition. Peptidyl trifluoromethyl ketones are slow-binding inhibitors of subtilisin as shown in Figure 1 for the inhibition of subtilisin Carlsberg by Z-LLF-CF_3. The constants K_i and k_{on} are summarized in Table 1, along with data for inhibition of subtilisin BPN′ by Z-AAPF-CF_3 (Jackson & Fersht, 1993). The slow binding is a result of the very low effective concentration of the ketone inhibitor that exists predominately as the hydrate in aqueous solution. NMR spectra in the presence of the TFMK inhibitors were acquired under conditions where the inhibitor was in excess over the enzyme concentration.

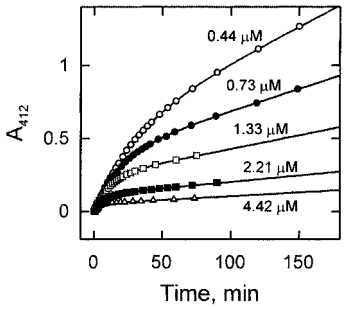


FIGURE 1: Progress curves for the inhibition of subtilisin Carlsberg by Z-LLF-CF_3. The hydrolysis of sAAPFpNA was monitored at 412 nm in the presence of 0.5 nM enzyme and the indicated concentrations of inhibitor at 25 °C and pH 7.5 maintained with 100 mM potassium phosphate. The data were fit according to eq 2.

Table 1: Summary of Kinetic and Equilibrium Constants for Inhibition of Subtilisins by Peptidyl TFMK Inhibitors

enzyme	peptide	K_i (nM)	$k_{on} \times 10^{-3}$ (M^{-1} s^{-1})
subtilisin Carlsberg[a]	Z-LLF-CF_3	40	3.5
subtilisin Carlsberg[a]	Z-LAF-CF_3	15	3.3
subtilisin BPN′[b]	Z-AAPF-CF_3	20	0.53

[a] 100 mM potassium phosphate (pH 7.5) and 0.05% Tween. [b] 100 mM Tris-HCl (pH 8.6) and 0.05% Tween (Jackson & Fersht, 1993).

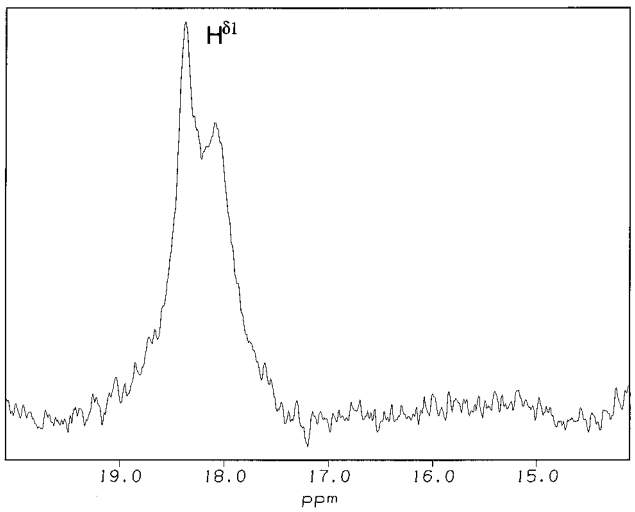


FIGURE 2: Downfield region of the ^1H spectrum at 500 MHz of subtilisin Carlsberg (2 mM) at 11 °C and 50 mM MES, (pH 5.5). The peaks at 18.4 and 18.1 ppm arise from the imidazolium Nδ1 proton of His 64. Data were acquired in 95% H_2O using a jump−return sequence to achieve selective excitation. The block size was 4096 complex points, the spectral width 16 129 Hz, and the postacquisition delay 200 ms, and 5000 scans were collected.

^1H NMR. At 500 MHz, the most downfield ^1H NMR resonances of subtilisin Carlsberg have chemical shift values of 18.4 and 18.1 ppm at pH 5.5 (Figure 2). These resonances have been assigned to the His 64 Nδ1 imidazolium proton (House et al., 1993). At 750 MHz, the two peaks appear to collapse into a single broad resonance (Table 2). At pH 7.5, a second downfield resonance is observed at 15.0 ppm with an intensity about equal to the peaks at 18.4 and 18.1 ppm (data not shown). The resonance at 15.0 ppm also arises from the Nδ1 proton of His 64; these resonances are in slow exchange and were assigned to His 64 in the imidazolium form (18.4 and 18.1 ppm) and imidazole form (15.0 ppm) of the active site histidine (House et al., 1993). The downfield resonance is also observed in subtilisin BPN′, as

Table 2: ^{1}H NMR Chemical Shifts and Fractionation Factors of the His 64 Resonances in Subtilisin Proteases[a]

enzyme	proton (N type)	inhibitor	pH	^{1}H chemical shift (ppm)	D/H fractionation factor (ϕ)
Carlsberg	His 64 (α+)	none	5.5	18.1, 18.4	0.55 ± 0.04
Carlsberg	His 64 (α+)	none	5.5	18.3[b]	0.63 ± 0.04[c]
Carlsberg	His 64 (α+)	none	6.0	18.1, 18.4	0.68 ± 0.04
BPN′	His 64 (α+)	none	6.0	18.0	ND
B. lentus	His 64 (α+)	none	6.0	18.0	ND
Carlsberg	His 64 (α+)	Z-LLF-CF_3	6.5−10.6	18.8	0.85 ± 0.05[d]
Carlsberg	His 64 (α+)	Z-LLF-CF_3	6.5−10.6	15.1[e]	ND
Carlsberg	His 64 (α+)	Z-LAF-CF_3	7.5	19.0	ND
BPN′	His 64 (α+)	Z-LLF-CF_3	7.5	18.6, 18.8[f]	ND
B. lentus	His 64 (α+)	Z-LLF-CF_3	5.5	18.8	ND
Carlsberg	His 64 (α)	none	9.5	15.0[b]	1.2 ± 0.3[c]

[a] Data were acquired at 11 °C and 500 MHz unless otherwise noted. The chemical shifts and fractionation factors refer to the Nδ1 proton unless otherwise noted. [b] Data were acquired at 5 °C and 750 MHz. [c] Values are the average (±standard errors) according to eq 5 determined using a jump−return or 1−1 sequence. [d] pH 7.5. [e] Resonance tentatively assigned to the Nϵ2 proton of His 64. [f] Two peaks are observed for subtilisin BPN′ complexed with Z-LLF-CF_3, consistent with separate populations that interconvert slowly on the NMR time scale.

seen previously (Robillard & Shulman, 1974). In subtilisin from *B. lentus*, a broad signal is also seen at ~18 ppm at pH 6.0 (Table 2). The low-field chemical shifts reported here for subtilisin proteases of widely differing sequence (Table 2) are unlikely to result from protons other than Nδ1 of the catalytic histidine (Robillard & Shulman, 1974; Bachovchin, 1985).

When subtilisin Carlsberg was complexed with Z-LAF-CF_3 at pH 7.5, a more Lorentzian resonance at 19.0 ppm was observed (Table 2). We assign this peak to the Nδ1 of positively charged His 64 in analogy to the ^{1}H NMR spectrum of TFMK-inhibited chymotrypsin (Liang & Abeles, 1987).[3] The catalytic histidine is positively charged in complexes of human leukocyte elastase (Stein et al., 1987), chymotrypsin (Brady et al., 1989), and subtilisin BPN′ (Jackson & Fersht, 1993) with peptidyl TFMKs.

Figure 3 shows that the chemical shift of the Nδ1 proton of subtilisin Carlsberg in a complex with Z-LLF-CF_3 at 18.8 ppm is independent of pH between pH 6.5 and 10.6, and the resonance broadens slightly with increasing pH. The invariance of the chemical shift of the low-field ^{1}H NMR resonance of subtilisin Carlsberg complexed with Z-LLF-CF_3 up to pH 10.6 indicates that the pK_a of 6.9 of His 64 Nϵ2H in the free enzyme is increased by over 4 pK units upon formation of the hemiketal in Chart 2.

An additional very broad resonance is present at ~15.1 ppm. Since the K_i of Z-LLF-CF_3 is 40 nM and this inhibitor is in 2-fold excess, this resonance must originate from inhibited, not free, enzyme. Moreover, the ratio of integrals for the peak at 15.1 ppm to the peak at 18.8 ppm is 0.5 at pH 7.5 and increases only to ~0.6 at pH 10.6. The lack of variation with pH indicates that the resonance at 15.1 ppm cannot be assigned to the Nδ1 proton of neutral His 64.[4] We note that trypsin and chymotrypsin exhibit two resonances between 16 and 18 ppm in complexes with boronic acid analogs of peptides and that the resonances have been assigned to the Nδ1 and Nϵ2 protons of the catalytic histidine (Hedstrom et al., 1994; Zhong et al., 1995). Therefore, we tentatively assign the resonance at 15.1 ppm to the His 64 Nϵ2 proton of inhibited subtilisin Carlsberg (Chart 2). Similar results for subtilisin BPN′ and subtilisin from *B. lentus* (Figure 4A) with Z-LLF-CF_3 were also observed and are summarized in Table 2.

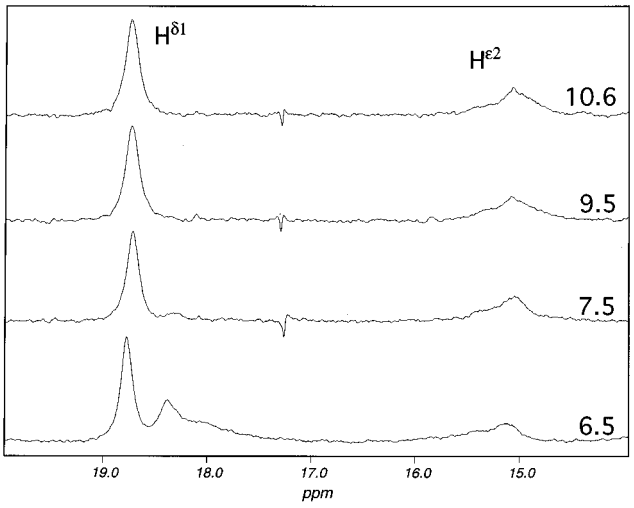
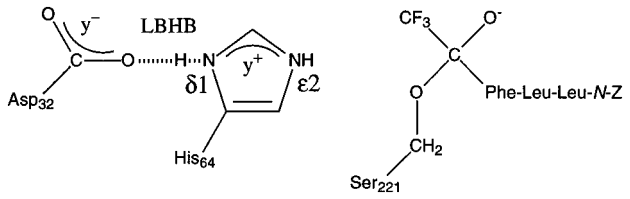


FIGURE 3: Downfield region of ^{1}H NMR spectra at 500 MHz of subtilisin Carlsberg (1.5 mM) complexed with Z-LLF-CF_3 at 11 °C, with 50 mM MES (pH 6.5), 100 mM potassium phosphate (pH 7.5), 100 mM CHES (pH 9.5), or 100 mM CAPS (pH 10.6, 0.02% sodium azide, and 0.05% Tween-20). The Nδ1 proton of His 64 has moved downfield and become sharper, relative to its position in the free enzyme (Figure 2). The spectra show little change between pH 6.5 and 10.6, other than broadening slightly as the pH is increased. Spectra were acquired in 95% H_2O using a jump−return sequence to achieve selective excitation. The block size was 4096 complex points, the spectral width 16 129 Hz, and the postacquisition delay 200 ms, and 7500 scans were collected.

Chart 2



When subtilisin from *B. lentus* is fully labeled with ^{15}N and complexed with Z-LLF-CF_3, 50 mM MES (pH 5.6), 1 mM $CaCl_2$ and 0.02% sodium azide, the resonance at 18.8

[3] A value of 18.9 ppm is observed with Z-LAF-CF_3 bound to chymotrypsin (J. Tobin, personal communication).

[4] By analogy to the free enzyme (House et al., 1993), one might (incorrectly) assign both the resonance at 18.8 ppm and the resonance at 15.1 ppm to the Nδ1 proton of His 64. The signal at 18.8 ppm would arise from positively charged His and the signal at 15.1 ppm from neutral His, and the two would be presumed to be in slow exchange. However, if the pK_a of His 64 were between 6.5 and 10.6 (Figure 3), the ratio of the integrals would change in this pH region, and if the pK_a were not, the ratio of the integrals would be far from unity. Since the ratio of the integrals is near 1 and constant with pH, the signal at 15.1 ppm cannot be assigned to the Nδ1 proton.

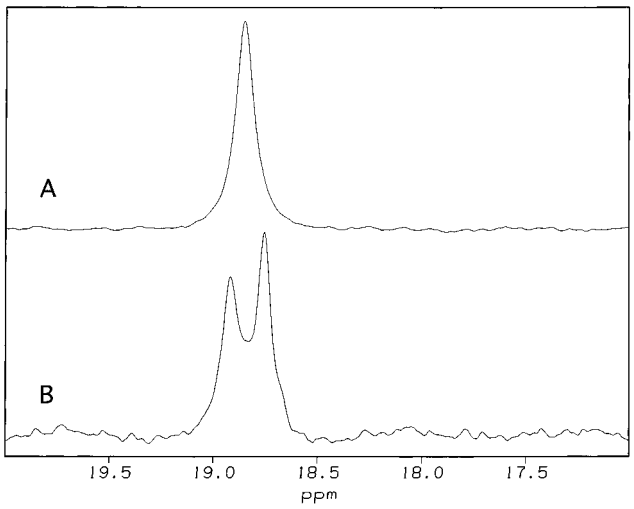


FIGURE 4: Downfield region of ^{1}H NMR spectra at 500 MHz of subtilisin from *B. lentus* (1.5 mM) complexed with Z-LLF-CF_3 at 11 °C in 50 mM MES (pH 5.6), 0.02% sodium azide, and 1 mM $CaCl_2$. Spectra were acquired in 95% H_2O with a jump−return sequence. The block size was 4096 complex points, the spectral width 16 129 Hz, and the postacquisition delay 200 ms, and 6000 scans were collected. (A) Unlabeled subtilisin from *B. lentus* gives a spectrum which is similar to that observed with subtilisin Carlsberg bound to Z-LLF-CF_3 (Figure 3). (B) ^{15}N-labeled subtilisin displays a splitting of $^1J_{NH} = 81$ Hz that arises from scalar coupling between the Nδ1 nitrogen and proton of His 64. Differential broadening of the two signals is apparent.

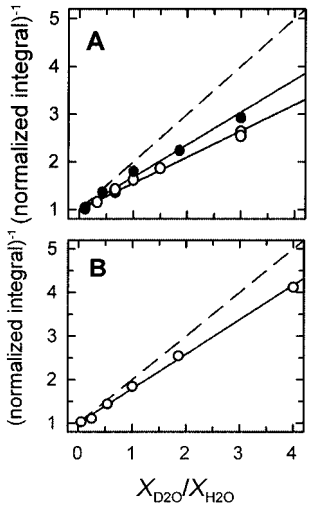


FIGURE 5: Determination of the fractionation factors (ϕ) for the Nδ1H···O hydrogen bond of the catalytic triad of subtilisin Carlsberg according to eq 5. The dashed lines correspond to a fractionation factor of unity. (A) The integral data for free subtilisin are normalized, and the slopes correspond to the fractionation factors at pH 5.5 (○, $\phi = 0.55 \pm 0.04$) and pH 6.0 (●, $\phi = 0.68 \pm 0.05$). (B) Normalized reciprocal plot as in panel A for subtilisin complexed with Z-LLF-CF_3 at pH 7.5 ($\phi = 0.85 \pm 0.05$).

ppm becomes a doublet with a one-bond ^{1}H−^{15}N coupling constant $^1J_{NH}$ of 81 Hz (Figure 4B). The two halves of the doublet are differentially broadened, due to interference between dipolar and chemical shift anisotropy relaxation (Guéron et al., 1983). ^{1}H−^{15}N HMQC spectra of this complex correlate the proton resonance at 18.8 ppm with a nitrogen resonance at 197.6 ppm relative to liquid NH_3 (data not shown).

Figure 5 shows the variation in signal area of the His 64 Nδ1 proton as a function of isotopic composition of the solvent for subtilisin Carlsberg at low pH and complexed with Z-LLF-CF_3. Values of the ^{1}H chemical shifts and isotopic fractionation factors as functions of pH and inhibitor are summarized in Table 2. The fractionation factor is about 0.6 at pH values of 5.5−6.0 where His 64 exists predominantly in the protonated form. The fractionation factor is 0.85 in the TFMK complex. These fractionation factors are somewhat lower than for fractionation factors of typical N−H bonds in water that lie between 1.0 and 1.2, with a few as high as 1.5 (Hibdon et al., 1992; Loh & Markley, 1994). The fractionation factor for the Nδ1 proton in the free enzyme increases to 1.2 ± 0.3 at pH 9.5 when His 64 is neutral (Table 2).

DISCUSSION

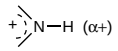
Recently, it has been proposed that a LBHB forms between the active site His-Asp pair in the transition state of peptide hydrolysis of serine proteases. A key tenet of this proposal is that the ground state does not have this LBHB (Frey et al., 1994). Since a LBHB is stronger than an ordinary H bond, its formation facilitates catalysis. Our study uses ^{1}H and ^{15}N chemical shifts, scalar coupling constants, and D/H fractionation factors to probe for the existence of LBHBs in subtilisin proteases.

Proton NMR of LBHBs. Proton chemical shifts of 16−20 ppm are considered evidence for the existence of LBHBs (Hibbert & Emsley, 1990). The magnitude of the downfield shift of the Nδ1 protons of the catalytic histidines is difficult to explain other than through the existence of LBHBs. The random-coil chemical shifts of amide protons fall between 8.0 and 8.5 ppm, depending on the side chain (Wüthrich, 1986). Subtilisin from *B. lentus* has a total of six main chain amide protons out of more than 260 with chemical shifts greater than 10 ppm (Remerowski et al., 1994); ribonuclease H (to take another example) has only one main chain amide proton out of more than 150 with a chemical shift greater than 10 ppm (Yamazaki et al., 1993). Thus, the range of amide shifts in proteins does not suggest that the low dielectric environment of a protein *per se* commonly leads to large downfield shifts.

Relatively few chemical shift data exist for protons bound to the nitrogen atoms of imidazoles because they often exchange with solvent too quickly to be observed (*vide infra).* However, in stromelysin, several slowly exchanging protons are observed from neutral histidine residues that coordinate zinc. These resonate between 11.5 and 13.8 ppm (Gooley et al., 1993). In small molecules in organic solvents, imidazole and imidazolium NH protons fall in the range of 7−12 ppm and depend upon solvent (Tobin et al., 1995). When His 64 of subtilisin Carlsberg is neutral, the chemical shift of the Nδ1 proton is 15.0 ppm; this proton may be more downfield than those of stromelysin due to the presence of the H bond from Asp 32 in the subtilisins. The chemical shifts of the His 64 Nδ1 proton are considerably further downfield for TFMK-inhibited subtilisins and the free enzymes at low pH than for the free enzymes at slightly alkaline pH. While the influence on the chemical shift of the positive charge is difficult to know with certainty, we take 13 ppm as an estimate of the chemical shift of imidazolium NH protons; therefore, chemical shifts of ∼18.9 ppm represent a downfield perturbation of almost 6 ppm.

Imidazole and imidazolium NH protons often exchange too quickly to be observed in protein NMR (Gooley et al.,

Table 3: ^{15}N NMR Chemical Shifts of Histidines in Model Compounds and Proteins[a]

^{15}N type	chemical shift (ppm)	H bond effect (Δ ppm)	reference
	Model Compounds		
	166	+10[b]	Bachovchin, 1986
	175	+10[b]	Bachovchin, 1986
	248	−10[b]	Bachovchin, 1986
	Proteins		
His 64 Nδ1, subtilisin from *B. lentus*/Z-LLF-CF_3 (α^+)	197.6	+22.6	this work
His 57 Nδ1, α-lytic protease/AAPV-CMK (α^+)	182.1−186.9	+7.3 to +11.9	Tsilikounas et al., 1996
His 57 Nδ1, α-lytic protease (α^+)	184.2	+9.2	Bachovchin, 1986
His 57 Nδ1, α-lytic protease (α)	176.4	+10.4	Bachovchin, 1986
His 95 Nϵ2, triose phosphate isomerase (α)	162.3	−3.7[c]	Lodi & Knowles, 1991

[a] ^{15}N chemical shifts reported here are relative to liquid NH_3 (downfield positive) by subtracting values reported relative to 1 M HNO_3 (upfield positive) from 375.8 ppm (Levy & Lichter, 1979). [b] Maximum chemical shift difference for H bond formation from model compounds reported by Bachovchin (1986). [c] His 95 is located at the N terminus of an α-helix, similar to the case for subtilisin. This value reflects the presence of an α-helix dipole (Lodi & Knowles, 1993).

1993; Zhong et al., 1995), but protons in LBHBs can display slow exchange rates (Hibbert & Emsley, 1990). The Nδ1 protons of free proteases at low pH show some evidence of exchange broadening. For example, for subtilisin Carlsberg (Jordan & Polgar, 1981) and chymotrypsin (Markley, 1978), a higher temperature increases the exchange rate with solvent sufficiently so that the downfield resonance is lost. In subtilisin Carlsberg, the width at half-height of the lowest field resonance is about 85 Hz (Figure 3, pH 7.5) upon complexation with Z-LLF-CF_3 and is only slightly broader than resonances at 10−12 ppm. These observations imply a decrease in the exchange rate due to complexation of subtilisin with TFMKs. TFMK complexes of chymotrypsin (Liang & Abeles, 1987) as well as α-lytic protease with boronic acid analogs of peptides (Bachovchin et al., 1988) also show decreased proton exchange rates of the catalytic histidine. This behavior is consistent with decreased accessibility of this residue to solvent, inhibition of the exchange pathway by the negative charge in the complex, or an increase in the barrier to exchange due to a change in the nature of the hydrogen bond itself (Bachovchin et al., 1988). The exchange rate and exchange mechanism of the Nδ1 proton in chymotrypsinogen varies with pH in a way that is consistent with a LBHB mechanism (Markley & Westler, 1996).

^{15}N NMR Chemical Shift and Scalar Coupling Constant. The data on ^{15}N-labeled subtilisin from *B. lentus* complexed with Z-LLF-CF_3 indicate that the His 64 Nδ1 nitrogen and proton reside in an unusual environment. The ^{15}N chemical shift of His 64 Nδ1 (197.6 ppm) is about 20 ppm further downfield than those of model compounds (Table 3) and ∼10 ppm further downfield than any value previously reported for a $=NH^+$ nitrogen nucleus (also known as $\alpha+$) in a serine protease. Protonated nitrogen nuclei of neutral imidazoles (termed =NH or α nitrogen nuclei) have chemical shift values of about 163.6−167.5 ppm, when referenced to liquid ammonia (Bachovchin, 1986).[5] When the imidazole ring carries a positive charge, the $=NH^+$ nuclei typically resonate between 171.4 and 177.8 ppm. In proteins, hydrogen-bonding interactions also influence the ^{15}N chemical shift (Van Dijk et al., 1990). For example, the Nδ1 nitrogen of the catalytic His 57 in α-lytic protease is observed at 176.4 ppm when His 57 is neutral (type α) and 184.2 ppm when His is protonated (type $\alpha+$; Bachovchin, 1986). Strong hydrogen bonding from Nδ1H to the cognate Asp in α-lytic protease inactivated by a chloromethyl ketone has been invoked to explain the downfield values of 182.1−186.9 ppm (Tsilikounas et al., 1996).

The ^{15}N chemical shift in the complex between subtilisin and Z-LLF-CF_3 suggests that the Nδ1 nitrogen nucleus begins to resemble unprotonated nitrogen nuclei, i.e. that some fraction of proton transfer has occurred. The ^{15}N chemical shift can be used as a measure of the extent of proton transfer on the basis of the 73 ppm difference in the chemical shift between type $\alpha+$ and type β nitrogen nuclei (Table 3) corresponding to complete proton transfer (Bachovchin, 1986; Smirnov et al., 1996). The observed $\Delta\delta$ of 22.6 ppm is consistent with ∼30% proton transfer in the complex.

The low dielectric microenvironment surrounding the catalytic histidine (Russell et al., 1987) cannot account for the unusual NMR spectroscopic properties reported here (Shan et al., 1996). The chemical shifts of type α nitrogen nuclei move upfield (smaller chemical shift), while for type β (unprotonated) nitrogen nuclei, ^{15}N chemical shifts move downfield as the dielectric constant of the solvent is decreased (Schuster & Roberts, 1979), opposite of the direction of increasing H bonding (Table 3). Furthermore, the location of His 64 at the amino terminus of an α-helical segment (Goddette et al., 1992) is expected to deshield the ^{15}N nucleus (Lodi & Knowles, 1991), again opposite of the observed effect. Finally, the remaining histidines in subtilisin from *B. lentus*, both buried and solvent-exposed, have not been reported to show the unusual spectral properties of the catalytic histidine (Remerowski et al., 1994; Fogh et al., 1995).

Unlike ^{1}H NMR, ^{15}N NMR has not yet been commonly applied to characterize normal H bonds versus LBHBs. However, one can use recent NMR studies on intermolecular complexes between pyridine and carboxylic acids as a helpful interpretative guide. These studies indicate that the chemical shift of the nitrogen nucleus moves smoothly downfield as

[5] ^{15}N chemical shifts reported here are relative to liquid ammonia (downfield positive) (Levy & Lichter, 1979; Wishart et al., 1995) by subtracting values reported relative to 1 M HNO_3 (upfield positive) (Bachovchin, 1986) from 375.8 ppm.

one proceeds from pyridinium−carboxylate complexes through LBHB complexes to neutral adducts as the pK_a of the carboxylic acid is increased (Smirnov et al., 1996). We note that the low-field proton chemical shifts and the positive differences between ^{1}H and ^{2}H chemical shifts at intermediate pK_a values indicate the existence of LBHBs for these members of the series (Smirnov et al., 1996).

The ^{1}H−^{15}N coupling constant of 81 Hz is relatively small, compared with values for the catalytic histidine in α-lytic protease that generally fall between 90 and 100 Hz (Tsilikounas et al., 1996). A value for $^1J_{NH}$ of 80 Hz has been reported for Nδ1 α-lytic protease at pH 4.0; under these conditions, the histidine is protonated and the Nδ1 proton resonance is found at ∼17 ppm (Bachovchin, 1985). While it is difficult to compare coupling constants obtained under widely differing conditions, these results suggest that downfield proton chemical shifts are correlated with reduced values of $^1J_{NH}$. This correspondence was observed for LBHBs between pyridine and carboxylic acids in solution at low temperatures (Smirnov et al., 1996). We conclude that overall the NMR data are consistent with a LBHB in the complex shown in Chart 2.

LBHBs and D/H Fractionation Factors. The D/H fractionation factors of the Nδ1 proton in subtilisin Carlsberg at pH 5.5−6.0 and bound to Z-LLF-CF_3 (Table 2) are less than that of aqueous amines and alcohols, which have fractionation factors of 1.0−1.2 (Hibdon et al., 1992; Loh & Markley, 1994), and are only slightly smaller than fractionation factors for $N^+H\cdots{}^-O$ hydrogen bonds in water (Hibdon et al., 1992). The values of fractionation factors for protons involved in LBHBs are expected to fall below 0.5 when decreased stretching modes are considered (Hibbert & Emsley, 1990; Cleland & Kreevoy, 1994). However, the increases in the in-plane and out-of-plane bending vibrations accompanying H bond formation *increase* the fractionation factor (Edison et al., 1995) such that occasionally fractionation factors take on atypical values. For example, the fractionation factors of a small number of amide groups of staphylococcal nuclease are as low as 0.3 or as high as 1.5, and none is involved in a LBHB (Loh & Markley, 1994). We conclude that the fractionation factors reported in Table 2 are consistent with the presence of a LBHB *when the His 64 is protonated* but do not require it.[6]

At pH 9.5, the Nδ1 proton of the free enzyme (in which His 64 is neutral) resonates at 15.1 ppm. While this is more deshielded than most histidinyl protons, it is not within the range typical of LBHBs (Frey et al., 1994). The value of the fractionation factor (Table 2) also demonstrates that the Nδ1 proton is not in a LBHB, as required by the LBHB proposal (Frey et al., 1994).[7] In the transformation of the ground state to the transition state, the hydrogen bond between (neutral) His 64 and Asp 32 is converted to a LBHB as His 64 becomes positively charged. The formation of the LBHB brings about stabilization of the transition state, as was proposed for chymotrypsin (Frey et al., 1994). There is extensive kinetic and structural evidence for proton donation to the catalytic histidine in the rate-limiting transition state for peptide bond hydrolysis (Satterthwaite & Jencks, 1974; Schowen, 1988). This suggests that the properties of positively charged His 64 should serve as a good model for the catalytic transition state of the reaction. Furthermore, the inverse of the inhibition constants of peptidyl TFMKs correlate with the k_{cat}/K_m values of the corresponding substrates for serine proteases (Stein et al., 1987; Brady & Abeles, 1990) with slopes of 0.7−1.3. The value of the slope indicates that many of the interactions present in the transition state are also found in the inhibited enzymes. We conclude that our observations with the free enzyme at low pH and in TFMK complexes constitute indirect evidence for a LBHB in the transition state of hydrolysis for subtilisin.

The proposal that LBHBs facilitate catalysis by serine proteases is supported by the fact that the distance between Asp 102 and His 57 in complexes between chymotrypsin and TFMKs is unusually short, 2.5−2.6 Å (Brady et al., 1990), although uncertainties in crystallographic distances (Bott & Frane, 1990) suggest caution in the interpretation of X-ray data. A survey of the active site His Nδ1···Asp Oδ2 hydrogen bond lengths in serine proteases determined by X-ray crystallography shows that the distance varies from 2.7 to 3.0 Å in the free enzyme and is independent of pH (Lange et al., 1994).

Comparison with Chymotrypsin. The subtilisins and chymotrypsin (or chymotrypsinogen) display many similarities as revealed by ^{1}H NMR spectroscopy of Nδ1 H of the catalytic histidine. In the free enzyme at low pH, a resonance at ∼18 ppm with a fractionation factor well below unity is observed (Robillard & Shulman, 1974; Markley & Westler, 1996). At high pH, the resonance moves upfield by ∼3 ppm and the fractionation factor is increased to unity or greater (House et al., 1993; Markley & Westler, 1996; this study). In the presence of TFMKs, the shifts are 18.8 ± 0.1 ppm, and the resonance has sharpened relative to that of the free enzyme (Liang & Abeles, 1987).[3] Finally, the inhibitor raises the pK_a corresponding to the Nε2 proton of the catalytic histidine in both subtilisin and chymotrypsin by at least 3 pH units (Liang & Abeles, 1987; Jackson & Fersht, 1993; this work). In fact, the histidine residues in the two complexes are the two most basic histidine residues of which we are aware. The perturbation of the pK_a may be the result of the LBHB between the conserved His and Asp (Frey et al., 1994), the anionic oxygen in the inhibitor when it is complexed to the enzyme (Brady & Abeles, 1990), or both. The increase in pK_a is advantageous to catalysis (Schowen, 1988). That these similarities are observed despite the fact that the two enzymes have completely unrelated primary and secondary structures is remarkable (Matthews et al., 1977). Moreover, these facts suggest that the structurally conserved aspartate of the catalytic triad creates an unusual local environment for the histidine Nδ1 proton, since the overall environment is different for the two enzymes.

Implications of LBHBs for Catalysis. Although the data reported here add to a growing list of examples of LBHBs in the catalytic mechanism of serine proteases (Frey et al., 1994; Markley & Westler, 1996), the exact energetic

[6] We note that there is a decrease in the flexibility of His 64 upon protonation in subtilisin from *B. lentus* on the basis of the decrease in crystallographic *B* values (Lange et al., 1994), and this may be consistent with an increase in bending vibrations (stiffening) of the $N^+H\cdots{}^-O$ bond.

[7] It might be argued that the changes in chemical shifts and fractionation factors with changes in pH are brought about by alterations in the structure of the enzyme with pH. This seems unlikely in that there are no aromatic rings (which might give a ring current shift) within 7 Å of His 64 in the X-ray structure of subtilisin Carlsberg (Matthews et al., 1977). There are small changes in the conformation of the active site triad with pH in subtilisin from *B. lentus* (Lange et al., 1994).

advantage of LBHBs in catalysis is difficult to measure. Replacement of Asp 32 by Ala in subtilisin BPN′ results in a destabilization of the transition state for hydrolysis of sAAPFpNA by $\Delta\Delta G = RT \ln[(k_{cat})_{wild\ type}/(k_{cat})_{mutant}] = 6$ kcal mol^{-1} (Carter & Wells, 1987). However, this change may reflect a different catalytic mechanism (e.g. attack of hydroxide ion), different rate-limiting steps, or both. Some groups have questioned whether the formation of LBHBs in enzyme-catalyzed reactions is at all catalytically advantageous (Warshel et al., 1995; Scheiner & Kar, 1995). In principle, solvent kinetic isotope effects should provide evidence for low fractionation factors in the transition state for enzyme-catalyzed hydrolysis of peptide substrates (Stein et al., 1983; Schowen, 1988), but this view has been challenged (Chiang et al., 1995). Further kinetic and structural analysis of LBHBs in enzymes will be required to dissect the energetic advantage of LBHBs in catalysis.

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