Hydrogen Bonding to Active-Site Histidine in Peptidyl Boronic Acid Inhibitor Complexes of Chymotrypsin and Subtilisin: Proton Magnetic Resonance Assignments and H/D Fractionation

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Abstract: ^{1}H NMR chemical shift assignments were established for $N^{\delta 1}H$ (16.9 ppm) and $N^{\epsilon 2}H$ (16.1 ppm) of the active-center His57 for the complex of MeOSuc-Ala-Ala-Pro-boroPhe (BoroPhe) with chymotrypsin and for the $C^{\epsilon 1}H$ proton (9.2 ppm at low pH and 8.5 ppm at high pH) of His57 in uncomplexed chymotrypsin. The assignment for $C^{\epsilon 1}H$ corrects previous assignments and reveals an unusual environment of this carbon-bound proton. The relative NH assignments are reversed from the order of NH assignments previously found for α-lytic protease complexes with boronate inhibitors. Isotopic fractionation factors (H/D) were determined using ^{1}H NMR for hydrogen bonds to the active site histidine in BoroPhe complexes with chymotrypsin and subtilisin E, and for uncomplexed chymotrypsin. Measured fractionation factors accurate to about ±0.1 were 0.82 (pH 10) and 0.64 (pH 3) for the $N^{\delta 1}H$ proton of uncomplexed chymotrypsin. In the presence of BoroPhe at pH 6.5, the $N^{\delta 1}H$ fractionation factors were 0.65 for the chymotrypsin−inhibitor complex, and 0.53 for the subtilisin−inhibitor complex. Measurements for the $N^{\epsilon 2}H$ fractionation factor were 1.05 (uncomplexed chymotrypsin at pH 10), 0.93 (BoroPhe−chymotrypsin at pH 6.5), and 0.76 (BoroPhe−subtilisin at pH 6.5). Both model calculations of isotopic fractionation factors and experimentally determined inhibition constants were used in the analysis of the fractionation-factor results.

Introduction

Renewed concern for the contributions of hydrogen bonding to enzymic catalysis[1−11] has sparked recent interest in measurements of isotopic fractionation factors (H/D) for hydrogen bonding sites in proteins.[12−18] H/D fractionation by normal acids and bases in protic solvents reflects differences in the character of hydrogen bonds relative to hydrogen bonds in the bulk solvent.[19−21] The origins of the isotopic fractionation are linked to hydrogen bonding through changes in hydrogen bond strengths, the symmetry of hydrogen bonds, and librational motions (hindered rotations) of groups participating in hydrogen bonds.[22−28] Changes in the character of hydrogen bonds can therefore be detected from changes in H/D fractionation factors.

The ^{1}H NMR of histidine N−H protons at the active center of serine proteases provides a convenient means to determine

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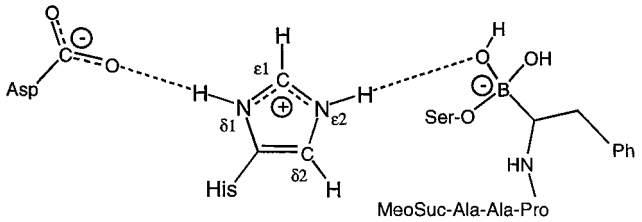

Figure 1. Depiction of some of the active-site groups in BoroPhe inhibited subtilisin or chymotrypsin. BoroPhe, MeoSuc-Ala-Ala-Pro-boroPhe, is the analogue of the tetrapeptide MeoSuc-Ala-Ala-Pro-Phe in which the C-terminal carboxyl group is replaced by the $-B(OH)_2$ group.

fractionation factors for sites thought to be critical to catalysis. In certain cases, the NMR signals can be observed which reflect protons in environments significantly deshielded from the typical environment of histidine residues.[29−35] Fractionation factors for these sites can be determined directly by monitoring the change in signal intensity with increasing proportions of deuterium oxide in the solvent. Under ideal circumstances, one might wish to compare isotopic fractionation factors for the free protease with those of an enzyme−transition state complex to learn about site-specific changes in active-center hydrogen bonds during catalysis. Indeed, kinetic solvent isotope effects provide valuable insights into the relative changes in fractionation factors during catalysis, but they do not provide direct information about specific sites. Instead, we sought to observe fractionation factors not for an enzyme−transition state complex, but for an enzyme−inhibitor complex (using tight-binding inhibitors which may resemble transition states or reactive intermediates) to learn about hydrogen bonds associated with binding a high-affinity ligand to the protease.

We chose to examine peptide−boronic acid complexes with chymotrypsin and subtilisin E in part because these inhibitor complexes exhibit observable NMR signals for both His57-NH (protonated imidazole ring) protons simultaneously near neutral pH. Because both fractionation factors for the complex can be determined in a single set of NMR experiments, their relative values are highly reliable. In previous reports,[34,35] we showed that the resonances for $N^{\delta 1}H$ and $N^{\epsilon 2}H$ of His57 in chymotrypsin can be observed over a wide pH range when complexed with peptide−boronic acid inhibitors. In these complexes, the His57 imidazole ring remains protonated, based on observation of two His57 NH NMR resonances, in buffers with pH at least as high as 10. For uncomplexed chymotrypsin, two NH His57 resonances can be seen below pH 5, a single NH signal can be seen at pH greater than 8, but near pH 7 exchange processes broaden the signals to such an extent that they cannot normally be observed.

We report here H/D fractionation factors determined for specific active-site hydrogen bonds involving histidine in chymotrypsin and in enzyme−inhibitor complexes of chymotrypsin and subtilisin E using the tight binding inhibitor MeoSuc-Ala-Ala-Pro-boroPhe (BoroPhe, Figure 1). Our results complement the recent measurements on related systems by Halkides, Wu, and Murray[15] and Lin et al.,[18] who studied complexes of subtilisin and chymotrypsin, respectively, with peptide−trifluoromethyl ketone inhibitors, and Markley and Westler,[13] who reported fractionation factors for chymotrypsinogen, the inactive proenzyme, or zymogen, of chymotrypsin.

In the course of completing our work, assignments of ^1H NMR signals for $N^{\delta 1}H$ and $N^{\epsilon 2}H$ of His57 were determined for BoroPhe−chymotrypsin. The corresponding assignments for BoroPhe−subtilisin were recently reported.[35] The chemical shift of chymotrypsin His57 $C^{\epsilon 1}H$ at pH 3 was reassigned using several different methods, correcting a long-standing incorrect assignment in the literature[18,36] and incidentally demonstrating that the environment around $C^{\epsilon 1}H$ of the active-site histidine is conspicuously different from the environment around $C^{\epsilon 1}H$ found in other histidine sites of the proteins.

Experimental Section

Sample Preparation. α-Chymotrypsin, three times crystallized type II from bovine pancreas, was from Sigma (St. Louis, MO). Subtilisin E and ^{15}N-labeled subtilisin E were expressed and purified as described previously.[35] MeoSuc-Ala-Ala-Pro-(D,L)boroPhe (BoroPhe) was synthesized as reported earlier.[37] All samples (except ^{15}N-labeled subtilisin E) contained 1−2 mM BoroPhe-inhibited or uncomplexed enzyme, DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt), as an internal reference, and a buffer: 50 mM K_2CO_3 at pH 10.0, 50 mM potassium phosphate at pH 6−7, 100 mM KCl at pH 3.0. The pH was measured at room temperature using an Orion microelectrode and the reading was corrected by 0.4 pH unit for D_2O solution.[38]

The active center histidine of ^{15}N-labeled subtilisin E in the presence of BoroPhe was observed at pD 6.9, 4 °C, by adding BoroPhe, freshly prepared in 99.9% D_2O at the same pH as of the protein sample, to 10 mg of labeled enzyme dissolved in D_2O. The mixture of the enzyme and inhibitor was incubated on ice for one-half hour before recording the spectrum to allow inhibition to proceed to completion. After addition of all of the BoroPhe, the same sample was used to study the pH dependence of the chemical shifts by adjusting the pH with dilute DCl or NaOD.

NMR Spectroscopy. NMR spectra were recorded at 4 °C on a Varian Inova 500 MHz spectrometer with a 5 mm triple resonance $^{15}N/^{13}C/^1H$ probe. ^1H chemical shifts were referenced to internal DSS. All NMR spectra were processed using the Varian VNMR software. The SS shaped pulse[39] was used in one-dimensional and one-dimensional NOE (nuclear Overhauser effect)[40] experiments to suppress the water resonance using the same parameters as reported earlier.[35] To detect the $C^{\epsilon 1}$ proton of histidine 64 in the ^{15}N-labeled subtilisin E (in 99.9% D_2O), a ^{15}N half-filter method[41] was used which selects carbon-bound protons and suppresses ^{15}N-bound proton resonances by turning them into heteronuclear multiple-quantum coherences. A 2 ms ^1H spin-lock period was used at the end of the pulse sequence to disperse antiphase components caused by imperfect pulses. The total delay time was 5.56 ms ($1/2J$), and ^{15}N was decoupled during the acqusition. The spectral width was 8003 Hz, and 8000 complex data points were collected and zero-filled to 8192 points. The signal-to-noise was enhanced by multiplication of a Gaussian window function prior to Fourier transformation. For each spectrum 2048 transients were collected. Spin-echo[52,53] and spin-lock[54] spectra were acquired using similar parameters to those used in the heteronuclear filtered experiment. The spin lock time was 50 ms to relax ^{14}N-bound proton signals.

For D/H fractionation factor measurements, a series of samples with different H_2O/D_2O mole fractions were prepared from H_2O and D_2O stock solutions.[13] Integrated intensities of the histidine N−H proton signals were determined relative to a proton peak in the aliphatic region

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of the spectrum. The integrated intensity of the reference aliphatic proton peak was found to be independent of the H_2O/D_2O mole fraction when compared to the integration of the internal DSS reference.

Determination of Inhibition Constants. Inhibition constants were determined for subtilisin E and bovine pancreas α-chymotrypsin using MeoSuc-Ala-Ala-Pro-(D,L)boroPhe (BoroPhe). Inhibition of enzyme activity was detected by spectrophotometrically monitoring the production of *p*-nitroaniline during the hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma) in the presence of varying concentrations of BoroPhe. Conditions for the inhibition assays were 50 mM potassium phosphate buffer at pH 6.5 (90 vol %) with dimethyl sulfoxide added (10 vol %). The same conditions were used for K_m determinations for the substrate in the absence of inhibitor. Temperature was controlled using a circulating water bath with a thermistor thermometer reading of 25.0 ± 0.1 °C. A Hewlett-Packard 8452a diode-array spectrophotomer was used for all measurements. The difference in absorbances at 390 (*p*-nitroaniline production) and 800 nm (no absorbance change) was recorded in time for each assay. Reactions were initiated by addition of enzyme solutions to the buffer containing inhibitor. For K_m determinations in the absence of inhibitor, initial velocities were determined from linear least-squares fits to the initial parts of progress curves. Progress curves for inhibition experiments were characteristic of time-dependent inhibition and were fit to an exponential-plus-line function using a least-squares method to obtain estimates of initial and final velocities. Because the enzyme concentrations used in the assays were similar in magnitude to the inhibition constants, a standard "tight-binding" inhibition equation (eq 1)[42] was used to obtain K_i from least-squares fits of either initial or final velocities vs inhibitor concentration. In eq 1, v is the reaction rate, v_o is the rate with no inhibitor present, f_E is the fraction of free enzyme (with no inhibitor present), e is the total enzyme concentration, and I_o is the total inhibitor concentration.

$$v = (v_o/2)([b^2 + 4K_i/(f_E e)]^{0.5} - b), \qquad b = K_i/(f_E e) + I_o/e - 1 \quad (1)$$

Results

Chemical Shift Assignments. A series of 1-D NOE, HMQC (heteronuclear multiple quantum correlation), and 2-D NOESY experiments was used previously to assign the low-field ^1H NMR resonances in the BoroPhe−subtilisin E complex at pH 6.5.[35] In this previous work, the histidinium ion N−H resonance at 17.4 ppm was assigned to the $N^{\delta 1}H$ proton; the resonance at 16.0 was assigned to the $N^{\epsilon 2}H$ proton. The assignments were based in part on a 1-D NOE experiment whereby the two N−H protons were selectively saturated. Saturation at 16.0 ppm produced two NOE signals in a region of the spectrum appropriate for the $C^{\epsilon 1}H$ and $C^{\delta 2}H$ protons (9.20 and 7.09 ppm). Only one NOE signal (at 9.20 ppm) was seen with saturation at 17.4 ppm. These results are clearly consistent with the assignments listed above for the N−H proton resonances.

The same 1-D NOE experiment was carried out with chymotrypsin (with no inhibitor present) at pH 3 and 10 to confirm the N−H proton assignments used earlier.[34] Figure 2 shows the results at pH 3 in spectra A and B. Saturation of the resonance at 13.3 ppm produced two NOE signals at 9.2 and 7.2 ppm; saturation of the 18.2 ppm resonance produced NOE signals at 9.2 and 10.5 ppm, similar to the recent report for peptidyl trifluoromethyl ketone-inhibited chymotrypsin18. The observations support assignment of 13.3 ppm to $N^{\epsilon 2}H$ and 18.2 to $N^{\delta 1}H$ because the two NOE signals in the upper spectrum correspond to C−H chemical shifts similar to what was reported for the BoroPhe-subtilisin at pH 6.5.[35] Unexpectedly, two NOE signals were also observed when the 18.2 ppm resonance (assigned to $N^{\delta 1}H$) was saturated (see spectrum B of Figure 2). The signal at 9.2 ppm is reasonably assigned to $C^{\epsilon 1}H$, based on the observation of the same signal seen when the 13.3 resonance

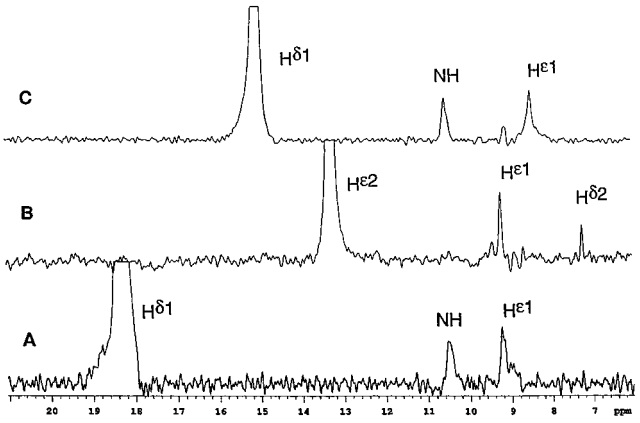

Figure 2. (A, B) One-dimensional NOE spectrum of chymotrypsin at pH 3.0, 4 °C. The largest resonance is the one irradiated; the two smaller signals in each plot are the major NOE effects. (C) One-dimensional NOE spectrum of chymotrypsin at pH 10.0, 4 °C.

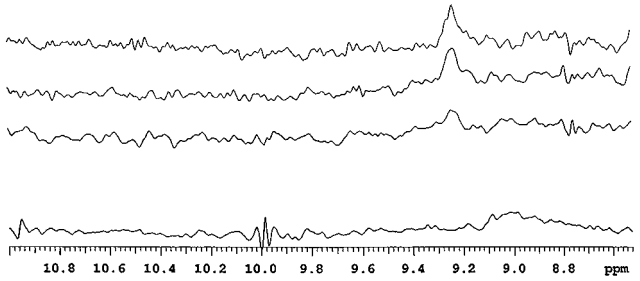

Figure 3. 1D spectrum with reverse half X-filter[41,44] of ^{15}N-labeled subtilisin E titrated with BoroPhe at 4 °C, pH 6.9, in 99.9% D_2O with 2048 scans each. The molar ratio of enzyme to inhibitor was 1:0, 1:0.4, 1:0.8, and 1:1.2 (from bottom to top).

was irradiated as described above. The 10.5 ppm signal is speculatively attributed to a backbone amide N−H proton. This conclusion is supported by the NOE experiment at pH 10 shown in spectrum C of Figure 1, in which the saturation of the protons giving rise to the single high-frequency (15 ppm) N−H resonance produced two NOE signals at 8.5 and 10.5 ppm. At pH 10, the $N^{\epsilon 2}H$ proton is absent, so the single high-frequency N−H signal is best assigned to the $N^{\delta 1}H$. The 10.5 ppm signal, which was also observed at pH 3.0, presumably results from a backbone proton. An examination of the X-ray crystal structure of chymotrypsin[43] revealed a likely assignment for this proton is the backbone amide proton of His57, which is 2.81 Å from $H^{\delta 1}$, similar to the distance between $H^{\delta 1}$ and $H^{\epsilon 1}$, 2.55 Å.

The unusual chemical shifts for $H^{\epsilon 1}$ of His57 in uncomplexed chymotrypsin at low pH (9.2 ppm) and high pH (8.5 ppm) inferred from the NOE studies prompted us to carry out further experiments to support these assignments. Direct observation of these proton resonances was not successful. Attempts at direct observation using spin-lock and spin-echo experiments with chymotrypsin, BoroPhe-chymotrypsin, and BoroPhe-subtilisin E all failed. However, using ^{15}N-labeled subtilisin E in a reverse half X-filter experiment (to suppress N−H signals),[41,44] a resonance at 9.2 ppm was observed as BoroPhe concentrations were increased (Figure 3). Changing the pH of the BoroPhe−subtilisin E solution (pH values of 4.81, 5.75, 6.80, 7.51, 8.22) did not change the chemical shift of this C−H resonance. The $N^{\delta 1}H$ and $N^{\epsilon 2}H$ proton resonances are also invariant over this range of pH for the BoroPhe−chymotrypsin complex.[34] Note that while measurements at pH 3 and 10 for uncomplexed

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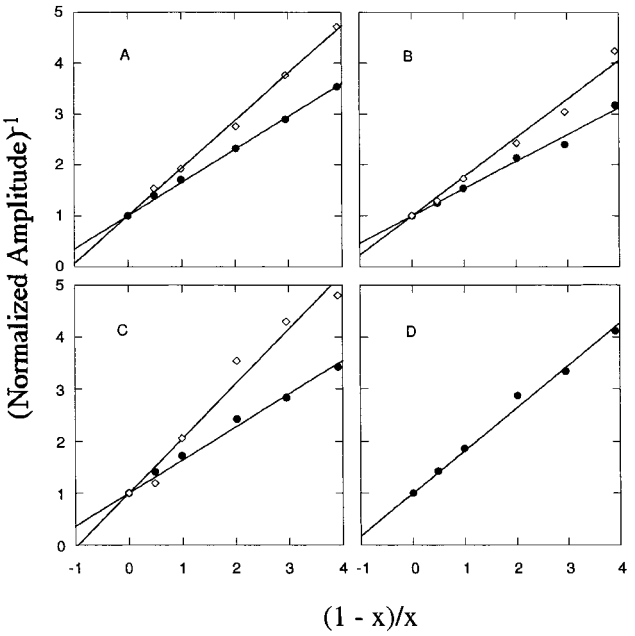

Figure 4. Fractionation factor determination for uncomplexed and BoroPhe-complexed serine proteases: (A) chymotrypsin−BoroPhe, (B) subtilisin E-BoroPhe, (C) chymotrypsin at pH 3.0, and (D) chymotrypsin at pH 10.0.

chymotrypsin are reliable, studies at intermediate pH values are difficult because the line widths of the resonances are very large. At intermediate pH values, we are unable to carry out meaningful NOE or fractionation factor experiments (see Figure 1a in a previous report[34]). Results on uncomplexed serine proteases are more difficult to obtain due to acid-denaturation of subtilisin, on one hand, and autolysis prominent under alkaline condition for both enzymes. This contrasts with the useful pH range for NMR experiments with the BoroPhe complexes of subtilisin and chymotrypsin. At both high and low pH values, there is evidence that multiple forms of the BoroPhe−enzyme complexes are present (see Figure 8 of the same report[34]).

A sense for error estimates in the chemical shift for the $H^{\epsilon 1}$ of His57 can be obtained by noting that measurements of the same samples on different days gave values (in ppm) of 9.240, 9.220, 9.259, 9.230, and 9.228 for chymotrypsin−boroPhe (mean = 9.235 ± 0.017 95% confidence limits) and 9.187, 9.203, 9.199, 9.191, and 9.203 for subtilisin E−boroPhe (mean = 9.200 ± 0.008 95% confidence limits).

Fractionation Factors. Isotopic fractionation factors were obtained from least-squares fits to eq 2, in which y is the value

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

of the integral of signals, C is a normalization factor, x is the mole fraction of H_2O, and ϕ is the D/H fractionation factor.[12] Figure 4 shows the data used to obtain the fractionation factors, and Table 1 summarizes the results along with the N−H proton chemical shifts. Least-squares estimates of the errors in ϕ ranged from ±0.01 to ±0.05. The smallest of these estimates of precision very likely overestimate the accuracy of the fractionation factors. In view of the range of normalization constants C (0.9 to 1.1) obtained by integrating the water peak in mixtures of H_2O and D_2O, a conservative estimate of the accuracy of the fractionation factors is ±0.10. Changes in fractionation factors, however, are expected to be accurate within the limits of the least-squares estimates of standard deviations (±0.01 to 0.05).

Table 1. ^{1}H NMR Chemical Shifts and Fractionation Factors (4 °C)

			$N^{\delta 1}H$		$N^{\epsilon 2}H$	
enzyme	inhibitor	pH	chemical shift, ppm	ϕ	chemical shift, ppm	ϕ
chymotrypsin	none	10	15.0	0.82 ± 0.02[a]	not seen	
chymotrypsin	none	3	18.2	0.64 ± 0.02	13.3	1.05 ± 0.05
chymotrypsin	BoroPhe	6.5	16.9	0.65 ± 0.01	16.1	0.93 ± 0.02
subtilisin E	BoroPhe	6.5	17.4	0.53 ± 0.02	16.0	0.76 ± 0.03

[a] See the Results section for a discussion of fractionation factor accuracy.

Inhibition Constants. Inhibition of both subtilisin E and chymotrypsin by BoroPhe was time-dependent. Analysis of the final velocities (steady-state rates after the exponential phase) as described in Experimental Procedures gave $K_i = 1.00 \pm 0.19$ nM for subtilisin E and 0.435 ± 0.036 nM for chymotrypsin. Over the nanomolar range of inhibitor concentrations used, the initial velocity (slope of the progress curve at the start of the experiment) was not dependent on BoroPhe concentration for subilisin E. Initial velocities for chymotrypsin were strongly dependent on BoroPhe concentration with the least-squares analysis providing $K_i = 2.46 \pm 0.90$ nM. In all cases, inhibitor concentrations used in the analyses refer to the sum of the diastereomeric components of (D,L)-BoroPhe. Previous work[37] has shown that for chymotrypsin, the D-isomer has a K_i at least 50 times greater than the L-isomer. Assuming that the stereochemical preference is similar for subtilisin E, our relative K_i values are useful measures of the relative binding strengths of the two enzymes for BoroPhe.

Discussion

NMR Studies of Active-Site Histidines in Serine Proteases. The NOE experiments reported here support the assignment of the His57 N−H proton signal at 18.2 ppm to $N^{\delta 1}H$, and 13.2 ppm to $N^{\epsilon 2}H$ in chymotrypsin at pH 3. Previous reports of the BoroPhe−subtilisin E and BoroPhe−chymotrypsin complexes,[35] as well as chymotrypsinogen at low pH,[13] also present assignments of the $N^{\delta 1}H$ proton at higher frequency than the $N^{\epsilon 2}H$ proton. Interestingly, these relative assignments are reversed in the spectra of complexes of BoroPhe with α-lytic protease,[45] which suggests that relative assignments of active-site resonances cannot be reliably transferred among serine proteases. New assignments must be established in every case if detailed, site-specific interpretations are needed. The methods reported here are the first successful ones for correlating the NH resonances with the CH resonances in the uncomplexed chymotrypsin, and should be applicable to other serine proteases.

Our assignments of the $N^{\delta 1}H$ and $N^{\epsilon 2}H$ proton resonances require a chemical shift of 9.2 ppm for the His57 $C^{\epsilon 1}H$ proton signal in chymotrypsin at pH 3. A chemical shift of 9.2 ppm for $H^{\epsilon 1}$ is distinctly different from the assignment used by other researchers (8.5 ppm) in previous investigations of serine proteases,[18,36] but it is similar to the value reported for chymotrypsinogen.[13] Detection of the 9.2 ppm signal required the use of ^{15}N-labeled subtilisin E in a 1-D X half-filter experiment. The absence of the signal in spin-lock and spin-echo experiments is presumably related to the fact that these experiments rely on different relaxation mechanisms for carbon-bound and ^{14}N-bound protons. The line width of the 9.2 ppm resonance in Figure 3 suggests that the relaxation time for $C^{\epsilon 1}H$ is unusual. The line width of the resonance is about 23 Hz corresponding a $T2$ relaxation time of 14 ms ($1/\pi\Delta V_{fwhh}$), shorter

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than the estimate of 21 ms (15 Hz line width) based on the molecular weight of 27 500 for subtilisin. The nearby C−H resonances in the same spectrum had the expected line width of 14 Hz, indicating that the $H^{\epsilon 1}$ of the active center histidine has an exceptional relaxation time, precluding detection using spin-echo and spin-lock techniques. The high chemical shift and unusual relaxation time may be a consequence of a significant interaction with a nearby carbonyl oxygen as suggested by Derewenda et al.[18,46] Previous assignments of low-pH His57 C−H proton resonances which differ from our assignments of 9.2 and 7.1 ppm might be reporting on proteolyzed enzyme, or perhaps on another conformation of the active-site histidine.

The resonance at 9.2 ppm has now been observed for complexes of chymotrypsin with peptidyl tryfluoromethyl ketones,[18] with BoroPhe, and in the absence of inhibitors at pH 3.0, as well as for chymotrypsinogen at low pH.[13] It seems that when His57 is protonated in all of these systems, the $C^{\epsilon 1}H$ proton always appears at 9.2 ppm.

Isotopic Fractionation Factors. The fact that the fractionation factors (Table 1) for active-site histidine $N^{\delta 1}H$ in the BoroPhe-inhibited enzymes and for uncomplexed chymotrypsin at pH 3 are much less than unity suggests that hydrogen bonds to the $N^{\delta 1}H$ proton are stronger than hydrogen bonds in the solvent water. Examples of fractionation factors in cases that clearly involve stronger hydrogen bonding than that found in solvent water include those for the hydronium ion protons and protons in the solvation shell of hydroxide ion (both have ϕ near 0.7)[19,20] and the 0.31 fractionation factor for bis(4-nitrophenolate) ion in acetonitrile.[47] Although a general and direct correlation between fractionation factors and hydrogen bond strength may be difficult to establish, model calculations[23,47] support the notion that lower fractionation factors can be expected in cases where the hydrogen bond heteroatom distances are decreased, perhaps as a consequence of stronger hydrogen bond formation.

The changes in fractionation factors shown in Table 1 are more easily explained in terms of changes in hydrogen bonding than are the changes in chemical shifts. The first entry in the table shows that $N^{\delta 1}$ has a modest preference, relative to that for water, for solvent-derived hydrogen over deuterium when the active-site histidine is unprotonated. Based on our estimates of the accuracy of the method (see Results), the fractionation factor for this site is 0.7−0.9. The results are not sufficiently accurate to claim that the $N^{\delta 1}$ proton is in a significantly stronger hydrogen bond than protons in the bulk solvent. However, upon protonation of His57 (the pH 3 results), the fractionation factor drops significantly from 0.82 to 0.64. Since the change in these values should be more accurate that the absolute values, the decrease is a clear indicator of a change in the hydrogen bonding, as expected with the charge generated on the histidine. The $N^{\epsilon 2}$ site, with a near unit fractionation factor, does not appear to be involved in hydrogen bonding distinguished from hydrogen bonds of the bulk solvent.

The BoroPhe−chymotrypsin complex at pH 6.5 has fractionation factors similar to those of the uncomplexed enzyme at pH 3. The similar values for the $N^{\delta 1}$ site arise presumably because His57 is protonated, even at pH 6.9, in the presence of the inhibitor.[34] The $N^{\epsilon 2}$ site, with $\phi = 1.05$, is not involved a hydrogen bond stronger than the bulk solvent. The relative values of the two fractionation factors should be highly accurate, and provide good support for the expectation that formation of stronger hydrogen bonds will be accompanied by decreases in ϕ. Halkides, Wu, and Murray[15] argued that for subtilisins inhibited by a trifluoromethyl ketone (*N*-carbobenzoxy-Leu-Leu-Phe-trifluoromethyl ketone) with a K_i of 40 nM, small changes in ϕ ($\phi = 0.85$) were consistent with the formation of very strong (low-barrier) hydrogen bonds. The increasing numbers of observations[16−18,47] of much lower fractionation factors argue against near complete compensation of changes in stretches by changes in bends, as postulated by Halkides, Wu, and Murray. Recent studies on chymotrypsin also using peptidyl trifluoromethyl ketones, for example, provided fractionation factors in the range of 0.32 to 0.43 for various inhibitors.[18]

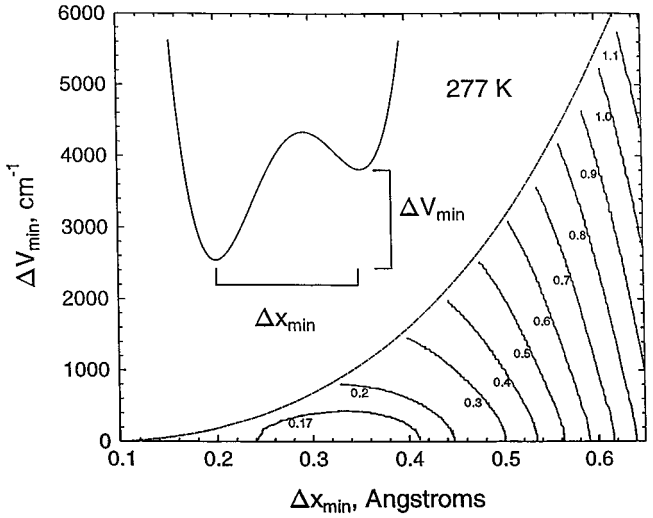

Figure 5. Fractionation factors from model calculations using a one-dimensional double-minimum potential for the hydrogen bond. The contours map out fractionation factors as a function of the two double-minimum features shown on the inset of the plot. The fractionation factors were calculated from vibrational partition functions for one-dimensional potentials. Following Kreevoy and Liang,[47] the reference model for the isotopic exchange expressed in a fractionation factor was taken as a simple harmonic potential corresponding to a frequency of 3500 cm^{-1} for a reduced mass of 1 atomic mass unit. The target models were double-minimum functions ($2V = f_2x^2 + f_3x^3 + f_4x^4$) describing the change in potential energy as the proton is displaced from the local maximum ($x = 0$). Vibrational energy levels were calculated using a variational method employing 40 harmonic basis functions[50,51] and reduced masses of 1 and 2 atomic mass units for the model isotopomers. Again following Kreevoy and Liang,[47] the constant f_4 was set to 18 mdyne/Å^3. The set of 900 fractionation factors used for the plot was generated by varying f_2 and f_3.

Model Calculations. The results of model calculations shown in Figure 5 provide indications of the types of changes in hydrogen bonds that are required for changes in fractionation factors. Isotopic fractionation factors were calculated from vibrational partition functions for one-dimensional, double-minimum potentials of the sort used by Kreevoy and Liang.[47] Briefly, vibrational energy levels were computed for a given model potential using masses for H and D and compared, through ratios of isotopic partition functions, with vibrational energy levels for a harmonic reference potential. The legend of the figure explains the details of the calculations. According to the Kreevoy and Liang model, changes in fractionation factors can be ascribed to changes in the symmetry of the hydrogen bond potential (ΔV_{min}) and a measure of the hydrogen bond heteroatom distance (Δx_{min}). Use of Δx_{min} is best illustrated by example: if the model is applied to an O−H−O hydrogen bond, at $\Delta x_{min} = 0.2$ Å, the heteroatom (O−O) distance can be taken as 1.0 + 1.0 + 0.2 = 2.2 Å, using 1.0 Å as a typical O−H bond length in the absence of a strong hydrogen bond.[47] The

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figure shows that models with large ΔV_{min} and long heteroatom distances have near unit fractionation factors, and models with small ΔV_{min} and short heteroatom distances have small fractionation factors. The origin of these trends lies in the loss of the difference in H vs D zero-point energy differences as the proton or deuteron becomes more centrally located and the asymmetric stretching motion of the hydrogen bond becomes less sensitive to mass.[23,48]

Limits on the hydrogen bond changes required to produce the changes in fractionation factors observed when BoroPhe binds to chymotrypsin and subtilisin can be obtained from Figure 5. Assuming the 0.82 value of ϕ for uncomplexed chymotrypsin ($N^{\delta 1}H$) at pH 10 is a reasonable estimate of the same ϕ at pH 6.5 for the free enzyme, the change in fractionation factor from 0.82 to 0.64 when BoroPhe binds to chymotrypsin would require at most a change in ΔV_{min} of 3000 cm^{-1} if the heteroatom distance of the hydrogen bond remained constant. A change of this size, moving along a vertical line on the figure, is equivalent to 8.6 kcal/mol or a change in ΔpK_a (for the hydrogen bond donors) of 6.3. Working horizontally across the figure gives limits on changes in heteroatom distances for fixed ΔV_{min}. For BoroPhe binding to chymotrypsin, the heteroatom distance would need to be compressed by at most 0.1 Å. Slightly greater changes in hydrogen bonding are predicted at $N^{\delta 1}H$ on subtilisin E on binding BoroPhe, assuming ϕ for uncomplexed chymotrypsin is similar to that for uncomplexed subtilisin at pH 6.5. A similar analysis applied to the relative fractionation factors for the two BoroPhe−enzyme complexes reveals that at both $N^{\delta 1}H$ (0.65 for chymotrypsin, 0.53 for subtilisin) and $N^{\epsilon 2}H$ (0.93 for chymotrypsin, 0.76 for subtilisin), the hydrogen bonds should be shorter and more symmetrical for the subtilisin E complex. For the BoroPhe−subtilisin E complex, both hydrogen bonds should be shorter by at most 0.05 Å and more energetically symmetrical by at most 1000 cm^{-1} (2.9 kcal/mol or a change in ΔpK_a of 2.1) than the hydrogen bonds in BoroPhe−chymotrypsin. The actual changes in hydrogen bond features are undoubtedly smaller than these limiting values.

Conclusion

The shorter, more energetically symmetrical, and presumably stronger hydrogen bonds in BoroPhe−subtilisin E are not manifested in tighter binding of the inhibitor to the enzyme. The inhibition constants (0.4 nM for chymotrypsin, 2.5 nM for subtilisin E) are similar in magnitude for the two proteases, but the binding is clearly tighter for chymotrypsin by 1.1 kcal/mol. The formation of the low-fractionation factor hydrogen bonds is better explained as a consequence of the tight binding (and concomitant increase in the active-site histidinium pK_a) of the peptidyl and boronic acid regions of the inhibitor, and not as a contributor to the tight binding. These serine proteases appear to be predisposed to form active-site hydrogen bonds distinquished by low fractionation factors at acidic pH or in the presence of certain inhibitors. The reality of such a predisposition in stable states of the enzymes suggests but does not demand a role for these hydrogen bonds in catalysis.[49] Evidence for the catalytic features of related hydrogen bonds stems from a transition-state analysis of kinetic solvent isotope effects.[11] Our work has direct relevance, however, to the general nature of protein−ligand interactions.

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