Proton Magnetic Resonance Studies of the Active Center Histidine of Chymotrypsin Complexed to Peptideboronic Acids: Solvent Accessibility to the N^{δ} and N^{ϵ} Sites Can Differentiate Slow-Binding and Rapidly Reversible Inhibitors[1]

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Received July 29, 1994[⊗]

Abstract: The proton chemical shift range 13−19 ppm downfield from silanes was reexamined for aqueous solutions of chymotrypsin in the absence and presence of boronic acid inhibitors. While ^{11}B NMR experiments had conclusively demonstrated that these inhibitors formed a tetrahedral complex at the active center and that Ser195 was required for the binding of these putative transition state analogs (Zhong, S.; Jordan, F.; Kettner, C.; Polgár, L. *J. Am. Chem. Soc.* **1991**, *113*, 9429−9435), there is important additional information available from observation of His57 imidazole NH proton resonances, clearly demonstrating the conditions under which such complexes exist. Supported by the assignments made for α-lytic protease (Bachovchin, W. W.; Wong, W. Y. L.; Farr-Jones, S.; Shenvi, A. B.; Kettner, C. A. *Biochemistry* **1988**, *27*, 7689−7697), information is presented on differential behavior of the $HN^{\delta 1}$ and $N^{\epsilon 2}H$ protons, depending on the strength of inhibition. Several complexes of a very potent, slow-binding inhibitor, MeO-Suc-Ala-Ala-Pro-BoroPhe, can be observed, some at strongly alkaline and acid conditions, whereas only one complex is observed with the rapidly reversible inhibitor MeO-Suc-Ala-Ala-Pro-BoroVal. At intermediate pH values, both of these inhibitors (a) must be bound to Ser195, since resonances corresponding to both $HN^{\delta 1}$ and $N^{\epsilon 2}H$ are clearly detectable, and (b) form complexes characterized by the same chemical shifts for their $HN^{\delta 1}$ and $N^{\epsilon 2}H$ resonances, the principal differences being reflected in the exchange rates of these protons, presumably due to their differential accessibility to water. At intermediate pH, the $HN^{\delta 1}$ and $N^{\epsilon 2}H$ resonances have pH-independent chemical shifts, providing evidence for an elevated pK of His57 on formation of the negatively charged boronates, as expected; the pK elevation parallels the strength of inhibition. While the peptideboronic acids appear to give rise to only one type of complex with chymotrypsin at intermediate pH values, 3,5-bis(trifluoromethyl)phenylboronic acid gives rise to two complexes, in accord with ^{11}B NMR studies, one Ser195, and the other probably His57-bound.

Introduction

The observation of His imidazole NH resonances in the active centers of enzymes was first reported by Bell Laboratories in the early 1970s for aqueous solutions of ribonuclease[2] and of serine proteases.[3] These broad, low-field ^1H NMR resonances were first detected using a continuous wave (CW) NMR technique and were assigned to the hydrogen-bonded proton attached to a His nitrogen ($N^{\delta 1}$) located between the imidazole and aspartate groups of the active site of serine proteases (the active center triad of chymotrypsin is comprised of Asp102, His57 and Ser195). Similar low-field proton resonances on trypsinogen, trypsin, subtilisin BPN′, and α-lytic protease were also studied in the low pH region (3.0−3.6). Later, the resonance was monitored[4] on porcine trypsin and on several protease−proteinase−inhibitor complexes, but difficulties were reported[5] in monitoring the reversible titration behavior of His NHs in the case of α-lytic protease.

An earlier publication from this laboratory[6] reported observation of this low-field ^1H NMR resonance in spectra of thiolsubtilisins and of complexes of subtilisin with boronic acids and with *N*-acetyl-D-tryptophan. The low-field resonance was observed clearly only for a (− + −) charge distribution at the catalytic center, i.e., $AspCOO^-HisH^+SerM^-$, where the serine OH is modified with a negatively charged moiety or is replaced by SH.

Insightful experiments from Markley's group[7] and Bachovchin[8] confirmed the assignment of these resonances to the His imidazole nitrogen-bound protons.

We reinvestigated the behavior of these protons to complement our own multinuclear NMR studies on serine proteases complexed to some transition state analogs, including boronic acids.[9] The ^{11}B NMR studies on the boronic acids, including a very potent peptideboronic acid, bound to serine proteases indicated that the enzyme-bound boron can be observed directly and that the boron has tetrahedral symmetry at the active center.[9c] The ^{11}B chemical shifts of the tetrahedrally bound boron atoms provided no insight concerning the differences

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[⊗] Abstract published in *Advance ACS Abstracts*, June 15, 1995.

(1) This research was supported by the Rutgers University Busch Biomedical Grant and by Ciba Geigy, Ardsley, NY. Based in part on the Ph.D. dissertation of S.Z., submitted to the Rutgers University Graduate Faculty, Newark, NJ, March, 1992. Presented in part at the Annual Meeting of the American Society for Biochemistry and Molecular Biology, Houston, TX, February 1992; Keystone Symposium on NMR in Biology, Taos, NM, March 1993; and Keystone Symposium on Proteases, Santa Fe, NM, February 1994.

(2) Patel, D.; Woodward, C.; Bovey, F. *Proc. Nat. Acad. Sci. U.S.A.* **1972**, *69*, 599−602.

(3) (a) Robillard, G.; Schulman, R. G. *J. Mol. Biol.* **1972** *71*, 507−511; (b) **1974**, *86*, 541−548; (c) **1974**, *86*, 519−540.

(4) Markley, J. L. *Biochemistry* **1978** *17*, 4648−4656.

(5) Markley, J. L. In *Biological Applications of Magnetic Resononance*; R. G. Shulman, Ed.; Academic Press: New York, 1979; pp 397−461.

(6) Jordan, F.; Polgár, L. *Biochemistry* **1981**, *20*, 6366−6370.

(7) Wu, X.; Westler, W. M.; Markley, J. L. *J. Magn. Reson.* **1984**, *59*, 524−529.

(8) Bachovchin, W. W. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7948−7951.

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between so-called "slow-binding" and classical rapidly reversible inhibitors. Having at our disposal boronic acid inhibitors of different strengths (ranging from millimolar to subnanomolar K_i values) prompted us to ask whether the behavior of the HisNHs in the active center mirrors the presence of inhibitors of such differing K_i values. For α-lytic protease, it was reported that slow-binding inhibitors gave rise to two clearly resolvable NH resonances,[10] whereas weaker, rapidly reversible inhibitors yield a single resonance near 15.5 ppm. To help interpret the data on inhibited chymotrypsin, we also report the spectra of the enzyme in the absence of inhibitor.

Using an optimized water suppression technique with a 1−1 binomial sequence,[11] we could observe the *entire titration curve of chymotrypsin* (never before reported by FT-NMR). Results on the same proton resonances have been reported for α-lytic protease,[10] subtilisin,[12] and trypsin,[13] some also in the presence of boronic acid inhibitors. Further characterization of these complexes is especially timely, in view of the recent proposal that these resonances represent hydrogens that participate in low-barrier hydrogen bonds.[14]

Abbreviations Used. $HN^{\delta 1}$ and $N^{\epsilon 2}H$ refer to the hydrogens on the imidazole of His57 (trypsin nomenclature) that point to the Asp102 and Ser195, respectively. MeOSuc-Ala-Ala-Pro-BoroPhe is the tetrapeptide that carries a methoxysuccinyl group at the amino terminus and $B(OH)_2$ in place of the carboxy-terminal COOH. MeOSuc-Ala-Ala-Pro-BoroVal carries the same connotation with Val as the C-terminal residue. Code for the resonances in the figures: $HN_0{}^{\delta 1}$, $HN_+{}^{\delta 1}$ for the 15 and 18 ppm resonances in uncomplexed chymotrypsin at pH above and below 7, respectively; $HN_{c1}{}^{\delta 1}$, $HN_{c1}{}^{\epsilon 1}$ for the 16 and 17 ppm resonances in the type 1 complexes (Ser195-bound), respectively, at intermediate pH values; $HN_{c2}{}^{\delta 1}$ for the 15.5 ppm resonance in type 2 complexes (bonding probably to His57).

Experimental Methods

NMR measurements were made at 400 MHz on a Varian VXR-400-89 unit using the software package supplied by the manufacturer. The 1−1 binomial water suppression pulse sequence[11] was used with a dedicated 5 mm $^1H/^{19}F$ probe for the proton experiments. Among the three pulse sequences studied, 11, 1331, and 1510, the 11 sequence gave the best signal-to-noise (S/N) ratio. In addition, zero filling was used to enhance the S/N ratio rather effectively. Typically, the free induction decay was truncated, but by using an appropriate weighting function, baseline distortions could be remedied. Typical instrument parameters used for acquisition and processing were as follow: acquisition time, 0.1−0.8 s; number of points, 3008; delay time, 1−3 s; spectral width, 14 000−15 000 Hz; pulse width, 16 μs (90°); number of transients, 400−4000, depending on need; zero filling with 65 536 points; FID phasing prior to Fourier transformation, 6.1; line broadening, 20−50 Hz. Some specific numbers are listed in some of the figure legends. Chemical shifts are reported in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonic acid, sodium salt (DSS). α-Chymotrypsin, three times crystallized type II from bovine pancreas (C-4129), was from Sigma (St. Louis, MO) or Worthington (Freehold, NJ). The concentrations reported in the figure legends refer to weighed amounts, and according to active site titrations, overestimate the concentration of active sites by at least 20%. MeOSuc-Ala-Ala-Pro-BoroPhe (BoroPhe) and MeOSuc-Ala-Ala-ProBoroVal (BoroVal) were synthesized as reported;[15] 3,5-bis(trifluoromethyl)phenylboronic acid (BTFPBA) was from Lancaster Synthesis Ltd. The pH of the solutions was measured on a Radiometer pHM62 standard pH meter using a Radiometer type G202 glass electrode. Some results have also been confirmed at 500 and 600 MHz. A 500 MHz 2D-NOESY experiment was performed to obtain spatial correlation between protons represented by the 16 and 17 ppm resonances and those corresponding to aliphatic and aromatic protons.

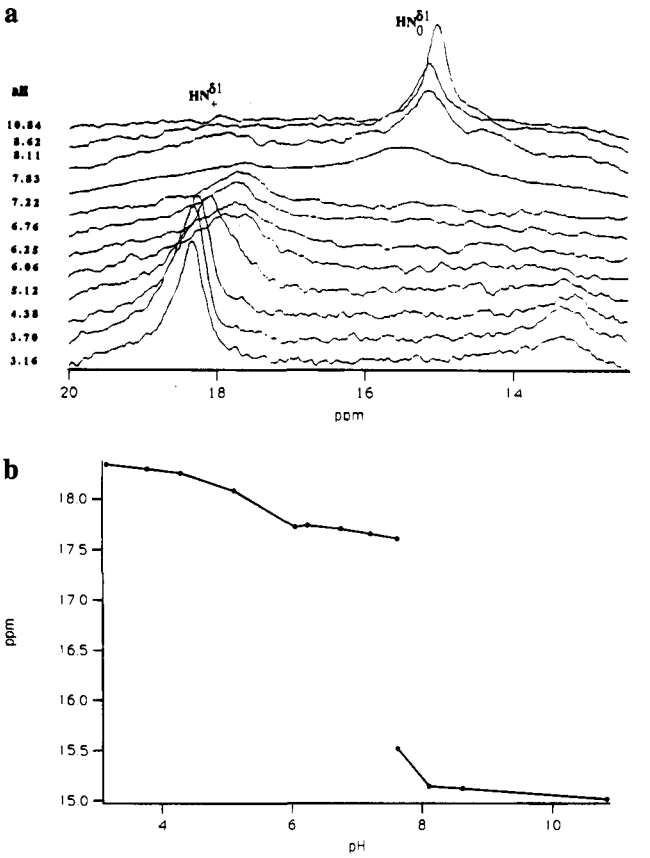


Figure 1. (a) 400 MHz ^1H NMR spectra of chymotrypsin at 5 °C and the indicated pH values. Concentration of chymotrypsin is 2.0 mM in 0.08 M phosphate in 80%H_2O/20% D_2O. Acquisition parameters: acquisition time, 0.1 s; delay time, 2 s; number of points, 3008; spectral width, 15 000 Hz; pulse width, 16 μs (90°); number of transients, 400; processing parameters: zero filling with 65 536 points; FID phasing prior to transformation, 6.1; GOF, 0.00; line broadening, 20 Hz. (b) Plot of the chemical shift (measured from internal DSS) vs pH.

Results

Uncomplexed Chymotrypsin. Chymotrypsin has excellent pH stability, especially under acidic conditions. The bell-shaped pH dependence of the kinetic parameters for chymotrypsin reflects two pK values: one near 7 has been assigned to the active center His, and the other at ~8.9 to the salt bridge between Ile16 and Asp194 that is formed as a result of zymogen activation.

Figure 1a shows the ^1H NMR spectrum of 2 mM chymotrypsin at 5 °C at various pH values, while Figure 1b provides the chemical shift vs pH plot. There are two distinct patterns in the acidic region. In the region below pH 5, the chemical shift is at ~18.2 ppm, the line shape is reasonably well defined (Lorentzian), and the line width is relatively narrow. Between pH 5 and 7, the chemical shift is between 18 and 17.6 ppm, the line shape is less well defined, while the line width is much broader. The appearance of the resonance at pH 6.06 may

(9) (a) Adebodun, F.; Jordan, F. *J. Am. Chem. Soc.* **1988**, *110*, 309−310; (b) *J. Cell. Biochem.* **1989**, *40*, 249−260. (c) Zhong, S.; Jordan, F.; Kettner, C.; Polgar, L. *J. Am. Chem. Soc.* **1991**, *113*, 9429−9435.

(10) Bachovchin, W. W.; Wong, W. Y. L.; Farr-Jones, S.; Shenvi, A. B.; Kettner, C. A. *Biochemistry* **1988**, *27*, 7689−7697.

(11) Redfield, A. G.; Kont, S. D.; Ralph, E. K. *J. Magn. Reson.* **1975**, *19*, 114−117.

(12) House, K. L.; Garber, A. R.; Dunlap, R. B.; Odom, J. D.; Hilvert, D. *Biochemistry* **1993**, *32*, 3468−3473.

(13) Tsilikounas, E.; Kettner, C. A.; Bachovchin, W. W. *Biochemistry* **1992**, *31*, 12839−12846.

(14) (a) Hibbert, F.; Emsley, J. *Adv. Phys. Org. Chem.* **1990**, *26*, 255−379. (b) Frey, P. A.; Whitt, S. A.; Tobin, J. B. *Science (Washington, D.C.)* **1994**, *264*, 1927−1930.

(15) Kettner, C. A.; Shenvi, A. B. *J. Biol. Chem.* **1984**, *259*, 15106−15112.

reflect a pattern of two overlapping resonances. The S/N ratio is sufficient to enable observation of the titration of the lower field resonance below pH 7 with an apparent pK of ~5.0. This behavior had been noted before.[3b] The temperature dependence of the spectra at pH 7.0 and 7.4 (Figure 2, supporting information) confirmed the slow exchange condition up to 30 °C. Similar behavior was observed on trypsin and subtilisin (unpublished) and on subtilisin by others.[12]

To estimate the number of protons represented by each resonance, we integrated the resonance at lower and higher pH values using the following protocol. A stock solution of chymotrypsin was prepared. The pH of one half of the solution was adjusted to 8.56, and that of the other half to 3.54. A spectrum was then accumulated at 5 °C, first at the higher pH, and then at the lower one using the *same external standard*, 0.05 M benzoic acid dissolved in carbon tetrachloride placed in a 5 μL pipet sealed at one end. The standard gives a rather *broad resonance centered at 13 ppm*. From the integration against the common external standard, it was concluded that under both acidic and alkaline conditions, we observe only one proton equivalent, likely pertaining to $HN^{\delta 1}$, as demonstrated by Bachovchin for α-lytic protease specifically enriched with ^{15}N.[8]

Chymotrypsin–Boronic Acid Complexes. For interpreting our observations, both the above results on chymotrypsin in the absence of inhibitors and the results on α-lytic protease, a serine protease with its single His located in its active center, are relevant. On α-lytic protease, a slow-binding peptideboronic acid inhibitor gave rise to two clearly resolvable, pH-independent, low-field ^1H resonances (16.5 and 16.0 ppm at pH 9.0 for BoroVal).[10] Such inhibitors were termed type 1 and denote[10] those boronic acids which are substrate analogs of serine proteases. Both resonances appeared as doublets with $^1J_{N-H}$ of ~90 Hz in the spectrum of the enzyme ^{15}N-labeled at both His nitrogens. With the $N^{\delta 1}$ singly labeled enzyme, the resonance farther downfield became a singlet, while the upfield resonance remained a doublet. The experiment unambiguously assigned the lower field resonance to $N^{\epsilon 2}H$ and the higher field one to $HN^{\delta 1}$. Type 1 complexes were suggested to involve a bond between the boron of the inhibitor and O^{γ} of Ser195. The weaker, rapidly reversible inhibitors gave rise to a single pH-independent resonance farther upfield (15.5 ppm) that becomes a doublet with $^1J_{N-H}$ of ~90 Hz in the spectrum of His-$^{15}N^{\delta 1}$-labeled enzyme. These were termed type 2 inhibitors and denote[10] those boronic acids which are not substrate analogs. The authors suggested that type 2 complexes involve formation of an adduct between $N^{\epsilon 2}$ of His57 and the boron of the inhibitors, while the $N^{\delta 1}$ of His57 remains hydrogen bonded to Asp102. Alternatively, in type 2 complexes, boron might be bound to both $N^{\epsilon 2}$ of the His57 and O^{γ} of the Ser195 simultaneously. We here report the behavior of chymotrypsin complexed to two peptideboronic acids: BoroVal (K_i in the micromolar range) and BoroPhe (K_i = 0.16 nM at 25 °C, pH 7.5; behaves as a "slow-binding" inhibitor).[15]

Chymotrypsin in the Presence of BoroVal. Figure 3 (supporting information) demonstrates that at pH 6.5 and 5 °C, on incremental addition of the BoroVal inhibitor to excess chymotrypsin, two new resonances appear in the spectrum, one broader at ~16 ppm, and the other narrower at ~17 ppm. A detailed pH titration of a mixture of the BoroVal with excess chymotrypsin at 10 °C is shown in Figure 4, clearly demonstrating that the free and inhibited enzyme are in slow-exchange with each other, since resonances corresponding to the free enzyme can be readily identified at both low and high pH (compare with Figure 1). As can be seen in Figure 4, above pH 8 there appears a shoulder on the resonance at 17 ppm *only*,

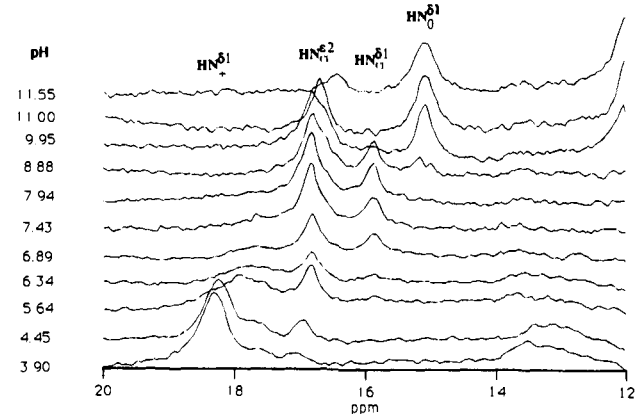
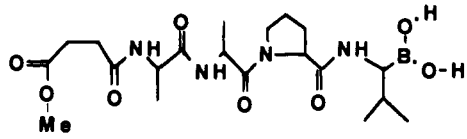


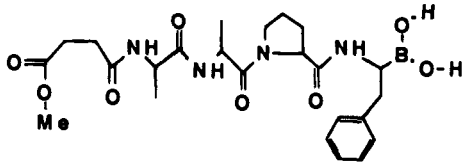
Figure 4. 400 MHz ^1H NMR spectra at 10 °C of a mixture of 1.65 mM BoroVal and 2 mM chymotrypsin dissolved in 0.08 M phosphate, containing 20% D_2O at the pH values indicated.

BoroVal



MeOSuccinyl-Alanyl-Alanyl-Prolyl-BoroVal

BoroPhe



MeOSuccinyl-Alanyl-Alanyl-Prolyl-BoroPhe

which at pH 11 becomes the only bound resonance. Also, the resonance at 16 ppm becomes visible near pH 7 and is again undetectable above pH 10. The exchange rate of the 16 ppm resonance with solvent is much greater than that of the 17 ppm one. The behavior of the resonances at 16 and 17 ppm is consistent with the bell-shaped pH–binding profile of boronic acids with serine proteases, which indicates optimal binding near pH 7 and diminished binding on either side of the optimum. This pH–binding profile reflects maximal affinity between trigonal, neutral peptideboronic acid and neutral His57, leading to histidinium cation and boronate anion in the complex. Figure 5 (supporting information) shows that in a mixture similar to that in Figure 4 at pH 6.36, as the temperature is increased from 5 to 30 °C, the resonance corresponding to free enzyme melts fastest, followed by the 16 then the 17 ppm resonance. Similar results are observed from the temperature dependence of such a mixture at pH 8.17 (Figure 6). There are several additional features in evidence. The exchange broadening is much greater in the free $HN^{\delta 1}$ resonance at ~15.0 ppm (the NH nearest to the Asp102 side) than in the BoroVal-bound species at ~16 and 17 ppm. The 16 ppm resonance is consistently smaller in area and "melts" at a lower temperature than the one at 17 ppm. Exchange of the protons in the complex with solvent protons is slow even at 40 °C, since both the 16 and 17 ppm resonances are still apparent. The "shoulder" on the 17 ppm resonance that becomes visible at about pH 8 broadens (or coalesces into the 17 ppm resonance) as the temperature is raised to 30 °C. A comparison with the temperature-dependent behavior at pH 6.36 (Figure 5, supporting information) is instructive, since the "shoulder" is not discernible at the lower pH. The appearance of this "shoulder" *above pH 8* indicates that there is a further change in electronic structure near the enzyme active center.

Chymotrypsin in the Presence of BoroPhe. On incremental addition of BoroPhe to chymotrypsin at pH 6.1 (Figure 7,

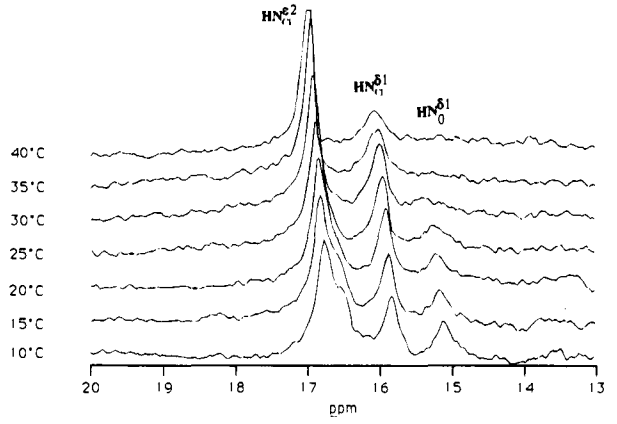


Figure 6. 400 MHz ^{1}H NMR spectra at pH 8.17 of a mixture of 1.65 mM BoroVal and 2 mM chymotrypsin dissolved in 0.08 M phosphate, containing 20% D_2O at the temperatures indicated.

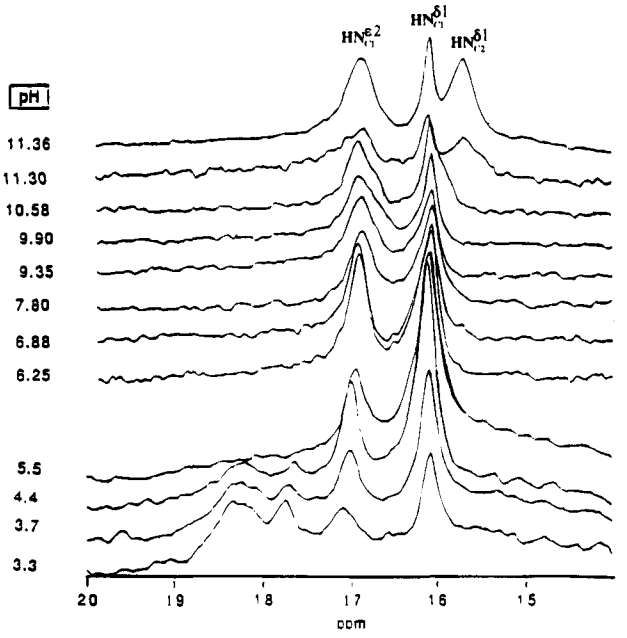


Figure 8. 400 MHz ^{1}H NMR spectra at 21 °C of a mixture of 1.67 mM BoroPhe and 2 mM chymotrypsin dissolved in 0.08 M phosphate, containing 20% D_2O at the pH values indicated. Acquisition parameters: acquisition time, 0.149 s; delay time, 3.00 s; number of points, 4480; spectral width, 15 000 Hz; pulse width, 15.7 μs (90°); number of transients, 2300. processing parameters: zero filling with 65 536 points; FID phasing prior to transformation, 6.1; line broadening, 30 Hz.

supporting information), there are two new resonances visible at 16 and 17 ppm, very similar in their chemical shifts to those observed with BoroVal. The spectrum demonstrates that the enzyme is in slow-exchange between the free and inhibitor-bound forms (as also observed by ^{11}B NMR), since free chymotrypsin is also in evidence when only a small amount of inhibitor is added. Figure 8 presents spectra of an equimolar chymotrypsin–BoroPhe mixture at 21 °C between pH 3.3 and 11.4. In the intermediate pH range (pH 5–10.6), the resonances at 16 and 17 ppm are present exclusively. This is consistent with the previously mentioned bell-shaped K_i vs pH profile. These spectra suggest that as the pH is increased, two additional complexes become observable. One of these is characterized by the "shoulder" upfield from the 17 ppm resonance that appears around pH 10–10.58 and is similar to that observed in the complex with BoroVal. The second one becomes detectable at pH 11.30 and has a chemical shift of 15.5 ppm, *different* from that at 15 ppm for unbound chymotrypsin at this pH. Since only one resonance is observed, whose chemical shift is upfield from those associated with the type 1 complex at 16 and 17 ppm, the likely explanation is that in this complex, $N^{\epsilon 2}$ is coordinated to the boron atom, forming a tetrahedral boronate, and the ring acquires only a partial rather than a full positive charge. The chemical shift of this new resonance is similar to those observed on the so-called type 2 inhibitors complexed with α-lytic protease[10] and those "nonspecific" boronic acid inhibitors associated with trypsin.[13] Another feature of the BoroPhe–chymotrypsin complex is the relative intensity of the resonances at 16 and 17 ppm. In the intermediate pH range, the intensities appear nearly equal. Also noteworthy is the observation of resonances different from those in the free enzyme near 18 ppm even at pH 3.3, signaling the presence of a complex different from that at intermediate pH. Since there are two resonances observed near 18 ppm at low pH, they may pertain to $HN^{\delta 1}$ in different environments (as is observed for the free enzyme) or perhaps to the $HN^{\delta 1}$ and $N^{\epsilon 2}H$ in the complex. The temperature dependence of the ^{1}H NMR spectrum at pH 4.2 (Figure 9, supporting information) showed that although the resonances at 16 and 17 ppm do not broaden significantly with elevated temperature, those near 18 ppm undergo significant line broadening with increasing temperature and are essentially unobservable at 40 °C. This result is consistent with a weaker complex and greater access to solvent exchange in the complex represented by the 18 ppm resonances.

Chymotrypsin in the Presence of BTFPBA: Evidence for Both Type 1 and Type 2 Complexes with the Same Inhibitor. According to ^{11}B NMR spectra reported from this laboratory[9c] and elsewhere,[16] BTFPBA forms two complexes with chymotrypsin. First, a mixture of 3 mM BTFPBA and 2 mM chymotrypsin was examined at 10 °C at pH 4.06, 5.10, 6.02, 7.05, and 8.0 (data not shown). While at pH 4 only the resonance corresponding to uncomplexed chymotrypsin is observed, at pH 5.10 two resonances appear in slow-exchange with that corresponding to free enzyme and are the only ones observed at or above pH 5.5. A comparison of spectra recorded at pH 5.6 and 7.0 showed these same two resonances. In comparison with the spectra of the BoroVal–chymotrypsin complex (the weaker peptideboronic acid inhibitor) at intermediate pH values, we assign the resonance at 17.2 ppm to $N^{\epsilon 2}H$ in a type 1 complex. The resonance corresponding to $HN^{\delta 1}$ should be at ~16 ppm in this complex, but it is either exchanging too fast or is masked by the large resonance at 15.6 ppm. The resonance at 15.6 ppm is assigned to the type 2 complex (Bachovchin's definition), characteristic of boronic acids that do not resemble the substrate. Both resonances have pH-independent chemical shifts and show differential line broadening with elevated temperature (Figure 10).

Discussion

Chymotrypsin Revisited. More previous NMR reports were concerned with inhibited serine proteases[3b,c,4,6,10,13] than with uncomplexed enzymes.[3b,c,17,18] The pH dependence of the chemical shifts in Figure 1 exhibits two transitions and implies two pK values: one near 5 and the other between 7 and 7.4. The coexistence of two resonances at pH 7 and 7.4, as well as a clear discontinuity in the titration curve between pH 7 and 7.4, suggests that the interchange between the protonated $HisH^+$ and neutral His^0 environments (represented by the 17.8 and 15.0 ppm resonances, respectively) is in the slow-exchange regime at this temperature. This is the pH region overlapping the apparent pK of 7, usually accepted as that pertinent to the active

(16) Baldwin, J. E.; Claridge, T. D. W.; Derome, A. E.; Schofield, C. J.; Smith, B. D. *Biochem. Med. Lett.* **1991**, *1*, 9–12.
(17) Markley, J. L.; Ibanez, I. B. *Biochemistry* **1978**, *17*, 4627–4640.
(18) Bachovchin, W. W. *Biochemistry* **1986**, *25*, 7751–7759.

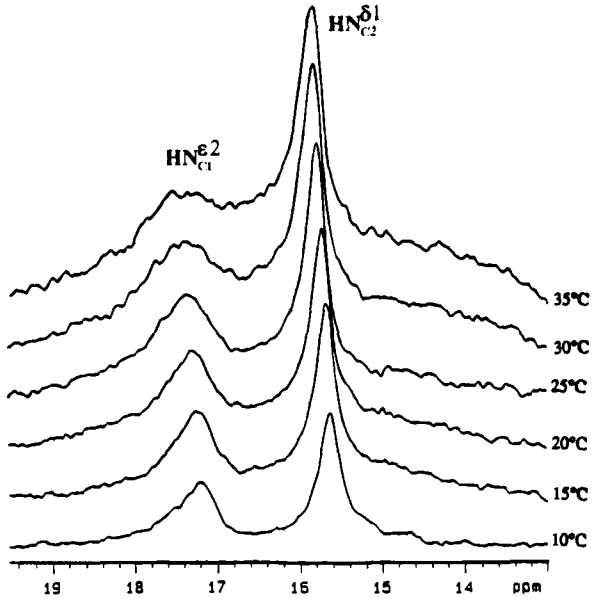


Figure 10. 400 MHz ^{1}H NMR spectra at pH 6.99 of a mixture of 3.0 mM BTFPBA and 2 mM chymotrypsin dissolved in 0.08 M phosphate, containing 20% D_2O at the indicated temperatures.

center His. Slow-exchange between conformations with protonated and neutral His57 is also seen on subtilisin and trypsin.

The magnitude of the apparent pK near 5 suggests an Asp or Glu side chain ionization. There results a small shielding of the resonance (~0.5 ppm) as the pH of the solution is increased. Ortiz et al. attributed a pK near 5.6 to the ionization of Asp102COOH in trypsin on the basis of a very different observation in inhibited enzyme.[19]

The pH-dependent behavior of the chemical shifts (Figure 1b) cannot be fitted to a theoretical curve using the three-site fast-exchange scheme in eq 1,

$$\delta = \delta_1[1] + \delta_2[2] + \delta_3[3] \quad (1)$$

where [1], [2], and [3] are the mole fractions of the species below pH 5, those protonated between pH 5 and 7, and neutral His above pH 7, and δ_1, δ_2, and δ_3 are the corresponding chemical shifts, respectively. One reason is that the transition near pH 7 is in the slow- rather than the fast-exchange regime. A further reason is that the two pH transitions among species 1, 2, and 3 are too close to each other, relative to the differences in chemical shifts of the proton in the three different protonation states. A series of theoretical curves were calculated to simulate the pH−chemical shift relationships, using experimental chemical shift data for δ_1, δ_2, and δ_3 in eq 1 and assuming fast exchange among the three species (S. Zhong, unpublished). It became evident both that the pK_a apparent in the acidic region will not be observed unless the difference between the two pK_a values is at least 3 units and that the chemical exchange rate of the observed protons cannot be explained by a fast-exchange mechanism. The theoretical plots suggest that the actual pK is at least 1 unit lower than the apparent pK of 5.0 inferred from the pH-dependent chemical shifts. Markley and Ibanez inferred pK values of 6.1 and 2.8 from the pH-dependent chemical shift of the resonance assigned to C2H of His57 in chymotrypsin.[17]

Kinetic Implications. Bachovchin[8] presented eq 2 to enable estimation of kinetic constants for exchange of these NH protons in the ^{1}H NMR spectra of uncomplexed serine proteases:

$$\Delta w = 4\pi(\Delta\nu_{ab})^2[P_a(1 - P_a)^2/k_{off}] \quad (2)$$

where Δw is the line width of the resonance under fast-exchange between species a and b, $\Delta\nu_{ab}$ is the chemical shift difference between protonated and neutral forms of His57 in hertz, P_a is the mole fraction of His57 in the imidazolium ion form, and k_{off} is the first-order rate constant for deprotonation of the imidazolium ion. With eq 2, one can estimate a k_{off} for deprotonation of the $HisH^+$ form of chymotrypsin as $\sim 8 \times 10^3$ s^{-1}. While on chymotrypsin, the first-order rate constants for acylation and deacylation of the enzyme have been shown to be much slower than this number,[20] for serine proteases with higher $k_{cat.}$ values, the conversion of the protonated conformer to the neutral one may become rate-limiting, rather than any step involving the substrate. A different explanation for the observed slow-exchange is based on the known aggregation of these enzymes at the high concentrations required for NMR studies.[21] It is conceivable that such aggregates must first disaggregate prior to proton exchange, and a slow disaggregation rate may lead to the apparent slow-exchange. The recent X-ray structure determination on savinase, a serine protease related to subtilisin, showed that (1) raising the pH from 6 to 10.5 results in significant shifts of the three active center side chains with respect to each other; (2) the Ser and His are hydrogen-bonded to each other at both pH values, and presumably, the hydrogen bond is formed between the $HisN^{\epsilon 2}H$ and $SerO^{\gamma}$ at lower pH but between the $N^{\epsilon 2}$ and the $SerO^{\gamma}H$ at pH 10.5; and (3) the imidazole ring tilts at pH 10.5.[22] Those structural alterations could account for the apparent slow-exchange between the two environments on the NMR time scale.

Peptideboronic Acid−Chymotrypsin Complexes. (i) Assignments. In a series of incisive studies, Bachovchin reported ^{15}N and ^{1}H NMR spectra for α-lytic protease specifically ^{15}N-enriched at both His57 nitrogens both in the absence[8,18] and in the presence of the peptideboronic acids here studied.[10] While the relative K_i values vis-à-vis the two inhibitors here studied are reversed between α-lytic protease and chymotrypsin (α-lytic protease binds BoroVal much more strongly), the assignments on α-lytic protease in the presence of its strong inhibitor should be usable to interpret our data with the resonances at 16 and 17 ppm assigned to $HN^{\delta 1}$ and $N^{\epsilon 2}H$, respectively. Preliminary 2D-NOESY experiments on the BoroPhe−chymotrypsin complex indicate that the 17 ppm resonance has several more cross-peaks in the aliphatic region than the 16 ppm one. An inspection of the X-ray structures of the α-lytic protease−BoroVal and α-lytic protease−BoroPhe complex[23] shows that the $N^{\epsilon 2}H$ makes many more close contacts (<5 Å) with the P1 and P2 side chains of the inhibitor than the $HN^{\delta 1}$, providing independent evidence consistent with assignment of the 17 ppm resonance to the $N^{\epsilon 2}H$. BoroVal has an approximately micromolar K_i, and the slow-binding inhibitor BoroPhe has a K_i of 0.16 nM at pH 7.5.[15] Both complexes exhibit both the 16 and 17 ppm resonances in the intermediate pH range, assigned to $HN^{\delta 1}$ and $N^{\epsilon 2}H$, respectively, and hence can be classified as type 1 complexes. The chemical shift of the resonance pertinent to $N^{\epsilon 2}H$ should be more affected by complexation, since this proton is closer to the boronic acid. However, the magnitude of the boronate-induced shift cannot be estimated for this proton, since no resonance corresponding to this proton has yet been identified in *any* uncomplexed serine protease. The chemical shift of $HN^{\delta 1}$ moves upfield ~2 ppm compared to the corresponding resonance below pH 7 in the uncomplexed enzyme. This shielding could have a contribution from a compression

(19) Ortiz, C.; Tellier, C.; Williams, H.; Stolowich, N. J.; Scott, A. I. *Biochemistry* **1991**, *30*, 10026−10034.

(20) Fersht, A. *Enzyme Structure and Mechanism*; W. H. Freeman and Co.: New York, 1985; pp 205−207.

(21) Aune, K. C.; Timasheff, S. N. *Biochemistry* **1971**, *10*, 1617−1622.

(22) Lange, G.; Betzel, C.; Branner, S.; Wilson, K. S. *Eur. J. Biochem.* **1994**, *224*, 507−518.

(23) Bone, R.; Frank, D.; Kettner, C. A.; Agard, D. A. *Biochemistry* **1989**, *28*, 7600−7609.

of the Asp102COO$^-$−His57HN$^{\delta 1}$ hydrogen bond, i.e., closer proximity of HN$^{\delta 1}$ to the negative charge at Asp, in addition to the shielding induced by the negative charge on the boronate. A different explanation is that the complexation *loosens* this hydrogen bond. This explanation is consistent with a recent hypothesis[14b] that the Asp-His HN$^{\delta 1}$ proton is part of a low-barrier, intermediate strength hydrogen bonding network whose strength is derived in part by the balancing of the pK values of donor (His57) and acceptor (Asp102). This postulate, based partly on a compilation of evidence for such hydrogen bonds by Hibbert and Emsley,[14a] would further suggest that with the elevated His57 pK value in the complex, the pK values are no longer balanced, and hence a weaker hydrogen bond and concomitant upfield chemical shift results. Our experiments showed that the HN$^{\delta 1}$ resonance invariably "melts" at higher temperatures in the presence of the inhibitor than in its absence. This is very likely due to stronger hydrogen bonds in the complex.

(ii) Intermediate pH. While ^{11}B NMR showed that the boronic acid is tetrahedrally bound to the active center of the enzyme for both complexes, it could not differentiate between the two potential covalent binding sites in the active center, Ser195 and His57. The observation of both HN$^{\delta 1}$ and N$^{\epsilon 2}$H resonances affirms that both inhibitors are bound to Ser195 rather than to His57 since the N$^{\epsilon 2}$ site is protonated in the complex. The persistence of the resonances at 16 and 17 ppm to at least pH 10 is consistent with elevation of the His57 pK value by the negatively charged boron covalently attached to Ser195 (as already noted in refs 6 and 10). Such pK elevation at His57 had earlier been reported by Liang and Abeles for the complex formed between chymotrypsin and a peptide trifluoromethyl ketone inhibitor[24] and was atttributed to formation of a hemiketal between Ser195 and the ketone, followed by ionization to an alkoxide and its stabilization in the oxyanion hole. That complex was characterized by a single resonance at 18.7 ppm, while the peptideboronic acid complexes give rise to two resonances at 16 and 17 ppm. A likely explanation, potentially of note for inhibitor design, is that while the boronates can form simultaneous hydrogen bonds to N$^{\epsilon 2}$H and the oxyanion hole, the trifluoromethyl ketone adduct can form only one of these hydrogen bonds. A similar pK elevation for His57 was also deduced for a different negatively charged transition state analog,[9b] monoisopropylphosphoryl-Ser195 chymotrypsin, and similar derivatives of trypsin and subtilisin.

(iii) Low pH. A significant difference between the behavior of the BoroPhe−chymotrypsin and BoroVal−chymotrypsin complexes is the presence of two resonances near 18 ppm at the lowest pH values studied in the spectrum of the BoroPhe−chymotrypsin complex *only*. As Figure 8 demonstrates, these resonances coexist with those pertaining to the HN$^{\delta 1}$ and N$^{\epsilon 2}$H with ionized Asp102COO$^-$ at 16 and 17 ppm, respectively, and are converted to them with an apparent pK near 5. All of these species are therefore in slow-exchange on the NMR time scale. The assignments are strongly supported by a comparison with the spectra of uncomplexed chymotrypsin, although the two-resonance feature is obscured by the near-fast-exchange properties in the case of uncomplexed enzyme. The nearly identical chemical shifts of the resonances associated with the inhibited complex (Figure 9, supporting information) and in the free enzyme under acidic conditions imply that the N$^{\epsilon 2}$ side has not yet developed negative charge, so that the binding is not due to formation of a tetrahedral covalent adduct to Ser. Rather, the complex may be due to formation of a Michaelis complex between BoroPhe and the enzyme through the S$_i$ subsites or to the presence of boron trigonally bound to O$^{\gamma}$ of Ser195, as found in the α-lytic protease−BoroPhe complex.[23]

(iv) High pH. The appearance of the shoulder only on the N$^{\epsilon 2}$H resonances in both complexes above pH 9 may be related to neutralization of the N-terminal α-ammonium at Ile16 (created by posttranslational zymogen activation) and concomitant scission of the salt bridge between Ile16 and Asp194, [25] which occurs at the pH at which this shoulder peak appears. That we do not observe this shoulder in the uncomplexed enzyme is expected, since we only observe the HN$^{\delta 1}$ in this case. BoroPhe forms a new complex at pH > 11 that is characterized by a chemical shift of 15.6 ppm. This chemical shift may signal conversion to a His57-bound complex.

The Case of BTFPBA. We assign the resonance at 17.2 ppm to that tetrahedral boronate characterized by a boron chemical shift of −18 ppm and the one at 15.6 ppm to the boronate with a ^{11}B chemical shift near −22 , on the basis of the relative intensities of the two tetrahedrally bound resonances in the ^{11}B NMR spectra of chymotrypsin.[9c] Chymotrypsin that had been inactivated with phenylmethanesulfonyl fluoride gave rise to only the −22 ppm ^{11}B resonance; hence, one tetrahedral boronate (^{11}B chemical shift of −18 ppm) was assigned to the serine-bound species, and the second one (^{11}B chemical shift of −22 ppm) was tentatively assigned to the tetrahedral boronate species that forms a Michaelis complex in the active center stabilized by the adjacent HisH$^+$.[9c] This possibility arises since the midpoint for trigonal−tetrahedral interconversion at boron for free BTFPBA is pH 7, two pH units lower than those for the peptideboronic acids. Based on its proton chemical shift, this second complex with a chemical shift at 15.6 ppm could represent a tetrahedral complex bound to His57 only or simultaneously to His57 and Ser195.[10,13] BTFPBA is the first inhibitor studied in our lab that appears to give rise to both types of complexes simultaneously. This enables us to deduce from the integrals that the type 2 complex is stronger than the type 1 complex for this inhibitor, as is also shown by the lower "melting" temperature of the type 1 complex represented by the 17.2 ppm resonance (Figure 10). Unpublished work by S. Zhong has established that these two complexes interconvert between themselves and with unbound inhibitor. We suggest further that the stronger complex may involve simultaneous binding of the boron to His57 and Ser195. This increased strength could signal the fact that for simultaneous binding to both side chains, a molecule of water must be eliminated.

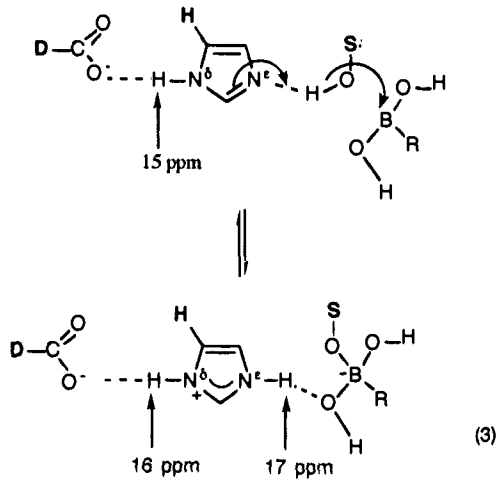
Implications of the Intensity Differences between the HN$^{\delta 1}$ and N$^{\epsilon 2}$H Resonances in the Boronic Acid Adducts. As a first approximation, the intensities of the HN$^{\delta 1}$ and N$^{\epsilon 2}$H resonances in the enzyme−inhibitor adduct should be similar, so that the observation of different intensities for these two resonances may seem paradoxical. There exist several mechanisms which can change the line width or intensity of the observed proton signals: ^{14}N−H quadrupolar relaxation, chemical exchange among different environments for the same proton, and proton exchange with solvent or other proton acceptors and donors. Given that the relative intensities of the HN$^{\delta 1}$ and N$^{\epsilon 2}$H resonances differ even for complexes involving the two peptideboronic acid inhibitors, it is unlikely that differential quadrupolar relaxation is responsible for the observations. Chemical exchange is a possible source of the observed behavior. Making the reasonable assumption that the two sites represented by the resonances at 16 and 17 ppm are equally populated, one can perform model calculations to compare with experimental data.[26] Equation 3 shows that the tetrahedral boronic acid is formed by nucleophilic attack of the Ser195O$^{\gamma}$

(24) Liang, T.-C.; Abeles, R. H. *Biochemistry* **1987**, *26*, 7603−7608.

(25) Henderson, R. *J. Mol. Biol.*, **1970**, *54*, 341−354.

(26) Bovey, F. A. *Nuclear Magnetic Resonance Spectroscopy*, 2nd ed.; Academic Press: New York, 1988; Chapter 5.

on the boron atom, with His57 acting as general base. If the



(3)

reaction is fast on the NMR time scale, one may expect to observe an averaged signal corresponding to the $HN^{\delta 1}$ with $\delta\varpi$ =16 ppm − 15 ppm, whereas the chemical shift of the resonance at 17 ppm remains relatively constant, while its intensity depends on the rate of proton transfer. This is not consistent with our results, since the intensity of the resonance at 16 ppm does not show a dependence on the concentration ratio of enzyme to inhibitor. If the exchange rate is slow on the NMR time scale, one may expect to observe three separate resonances at 15, 16, and 17 ppm, their intensities depending on the K_i and the initial concentrations of enzyme and inhibitor. Experiments for the BoroVal−chymotrypsin adduct in the intermediate pH range (Figure 6) indeed reveal such behavior. We therefore conclude that the exchange rate between free and inhibitor-bound enzyme is slow on the NMR time scale in the intermediate pH range (indeed at all pH values). Accordingly, equal intensities would be expected for the $HN^{\delta 1}$ and $N^{\epsilon 2}H$ resonances; hence, the difference in the observed intensities is most likely due to proton exchange. Possible sources of the HisNH proton exchange are Ser195 on the $N^{\epsilon 2}$ side, Asp102 on the $N^{\delta 1}$ side, and solvent water on both sides. Normally, imidazole NH protons in aqueous solution are hardly detectable in a ^1H NMR spectrum because of fast solvent exchange rate on the NMR time scale. The fact that the His57NH proton is hardly detectable in a ~50% D_2O solution is direct evidence for such solvent effects. An increase in temperature would enhance the exchange rate between solvent and the NH proton; therefore, one would expect to observe broadening and diminished signal intensity upon increasing the temperature. The water solvent might have different accessibility to the two NHs of imidazole. The $HN^{\delta 1}$ on the Asp102 side is believed to be buried in a hydrophobic environment;[27] therefore, it should be shielded from exchange with solvent. The $N^{\epsilon 2}H$ on the Ser195 side, on the other hand, is accessible to solvent in the free enzyme and should undergo rapid exchange with solvent. Indeed, it has not yet been detected, except in the boronate complexes. The water molecule required for the deacylation step is indicated to be present according to diffraction studies on the $N^{\epsilon 2}H$ side, and this may provide a specific exchange mechanism for $N^{\epsilon 2}H$.[28,29] The ability to observe $HN^{\delta 1}$ and $N^{\epsilon 2}H$ with a better S/N ratio in the presence of boronate inhibitors than in their absence suggests that the solvent exchange rate is diminished at least on the Ser side, but most likely on the Asp side as well. The relative size of the two resonances at intermediate pH suggests that $HN^{\delta 1}$ is more accessible to exchange broadening than the $N^{\epsilon 2}H$ in the chymotrypsin−BoroVal complex, while the two resonances have similar line widths in the stronger BoroPhe−chymotrypsin complex. That is, the slow-binding inhibitor makes the Asp−His interaction locus more shielded. The implication from the temperature and pH effects on the spectra is that the noncovalent interactions via long-range effects tighten the entire protein structure more in the chymotrypsin−slow-binding inhibitor complex. This is strikingly evident in the temperature dependence even at pH 4 (Figure 9, supporting information). The observation that resonances pertinent to the BoroPhe−chymotrypsin complex persist to a significantly higher temperature than those of the BoroVal−chymotrypsin complex also supports this conclusion. We conclude that differences in the relative intensities among the inhibitors are due to differential accessibility of solvent water to the two sites: in the absence of inhibitors, $N^{\epsilon 2}H$ is much more accessible than $HN^{\delta 1}$; in the chymotrypsin−BoroPhe complex, $N^{\epsilon 2}H$ is equally or more accessible than $HN^{\delta 1}$; in the chymotrypsin−BoroVal complex, $N^{\epsilon 2}H$ is less accessible than $HN^{\delta 1}$; and in the chymotrypsin−BTFPBA complex, $N^{\epsilon 2}H$ is much less accessible than $HN^{\delta 1}$.

Observation of a Resonance Near 13.3 ppm below pH 5. In addition to the resonances described, there is found a very broad and weak resonance below pH 5 with a chemical shift of 13.3 ppm, observed earlier in chymotrypsinogen[3b] but not in chymotrypsin. The presence of the resonance is quite clear in Figures 1, 4, and 9 (and in a number of other inhibited complexes; K. Haghjoo, unpublished), and its chemical shift, pH-dependent behavior, and line width are suggestive of a carboxylic acid proton, but it is as yet unassigned. This resonance may pertain to the side chain that affects the $HN^{\delta 1}$ resonance between pH 4 and 7, since below pH 5 this resonance coexists with the one at 18.2 ppm, which is assigned to the $HN^{\delta 1}$ proton. Since the resonance at 13.3 ppm is observed only under acidic conditions, and because of its chemical shift, the resonance is unlikely to pertain to $HN^{\delta 1}$ of the second His in the enzyme (the two His in chymotrypsin are at positions 40 and 57).

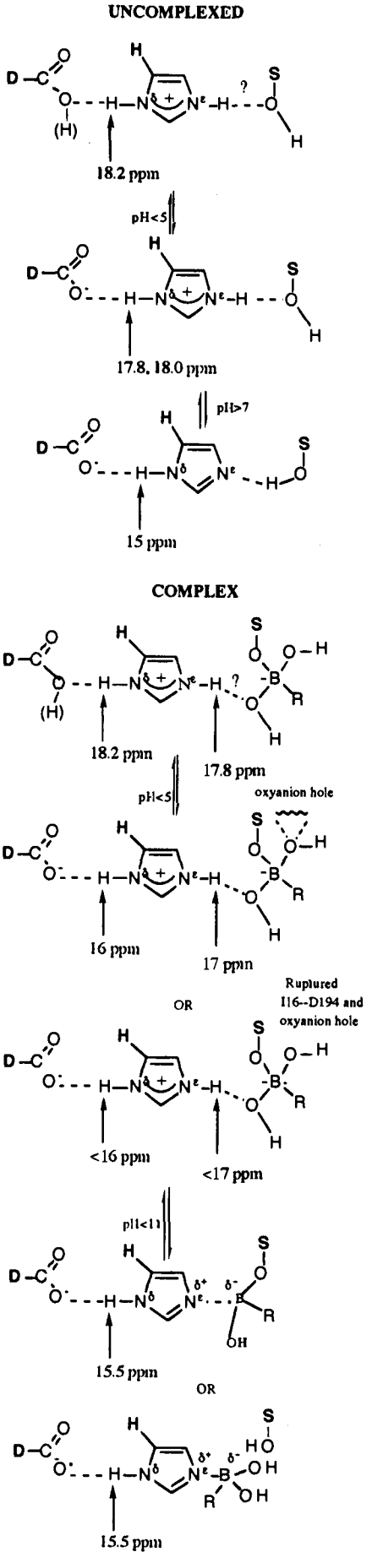
Conclusions

The similarities of the chemical shifts corresponding to $HN^{\delta 1}$, and $N^{\epsilon 2}H$ in chymotrypsin in its complexes with BoroPhe and BoroVal (indeed for one complex with BTFPBA as well) in the intermediate pH range suggest that their environments around His57 are similar, as was also observed for the ^{11}B chemical shifts in these complexes. The slow-binding inhibitor has its most dramatic effect on the dynamics/accessibility of the enzyme active center, compared to a weaker, rapidly reversible inhibitor. In addition, the results also show that with the stronger inhibitor, we can detect as many as four different complexes, depending on the pH.

The differential exchange rates observed for the $HN^{\delta 1}$ and $N^{\epsilon 2}H$ in the presence of various inhibitors may also be revealing that the interaction between inhibitor and the binding subsites of chymotrypsin is more important for the slow-binding inhibitor than for BoroVal or BTFPBA, since relatively, $N^{\epsilon 2}H$ is more accessible in the BoroPhe−chymotrypsin complex. It is important to point out that X-ray crystallographic studies failed to detect any changes in the structure of α-lytic protease complexed to the slow-binding or classical rapidly reversible peptideboronic acids, as here studied.[23] The dynamic differences so clear from the line shapes could not be detected using conventional X-ray methods and further underline the complementarity of the two techniques in protein structure determination.

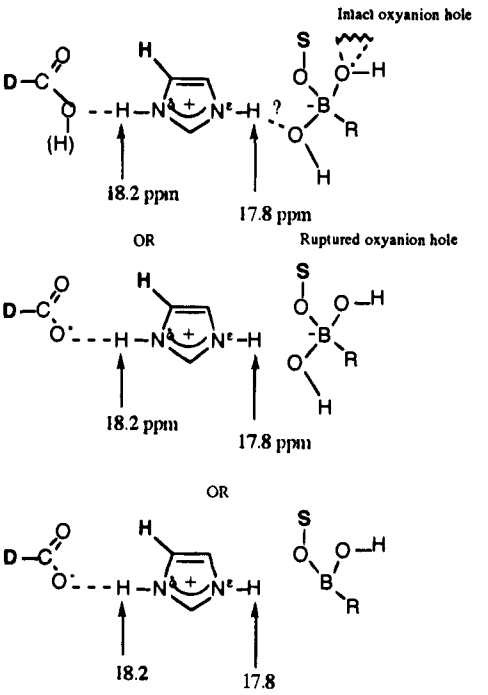
(27) Blow, D. M.; Birktoft, J. J.; Hartley, B. S. *Nature (London)* **1969**, *221*, 337−340.
(28) Perona, J. J.; Craik, C. S.; Fletterick, R. J. *Science* **1993**, *261* 620−621.
(29) Singer, P. T.; Smalas, A.; Carty, R. P.; Mangel, W. F.; Sweet, R. M. *Science* **1993**, *261*, 621−622.

Scheme 1



Scheme 1 summarizes the assignments deduced in this work. All observations made so far are consistent with shielding of

Scheme 2. Possible Structures for the Low-pH BoroPhe–Chymotrypsin Complex



$HN^{\delta 1}$ and $N^{\epsilon 2}H$ by neighboring negative charges and deshielding by imidazole protonation. As of now, we can only speculate about the side chain responsible for the apparent pK of 4–5, reflected in the behavior of chymotrypsin both in the absence and presence of BoroPhe. One possibility is the intervention of Asp102. A second possibility is drawn in Scheme 2, where the states of ionization of Asp102 and His57 remain the same from pH 3 to 10; it is the protonation of the Asp194–Ile16 ion pair that leads to alteration in the hydrogen bond strengths and the concomitant chemical shift changes. Irrespective of the source of the change, however, we again emphasize that in both chymotrypsin and trypsin, and in the boronate complexes here studied, there is clear evidence for this apparent pK. The chemical shifts of the low-field NH protons of His57 provide a description of the electron distribution, while the relative integrals and especially the temperature dependencies reflect the relative accessibility of the active center of serine proteases in the absence and presence of inhibitors. Our results underline the utility of Bachovchin's descriptions of two types of complexes and their assignments to Ser195 and His57. Which boronic acid falls into which of the two classes is harder to predict but can now be assigned with confidence with the accumulated data base on the bound chemical shifts. The three inhibitors here studied vary both so far as their K_i values are concerned (4 powers of 10) and also so far as slow-binding and rapidly reversible binding is concerned, yet all three appear to give rise to type I complexes.

Acknowledgment. The authors thank Dr. S. Pochapsky of Bruker Instruments for performing the 2D-NOESY experiment discussed.

Supporting Information Available: Figures 2, 3, 5, 7, and 9, illustrating 400 MHz ^{1}H NMR spectra of chymotrypsin solution (6 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of this journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

JA9425041