Nitrogen-15 NMR Spectroscopy of the Catalytic-Triad Histidine of a Serine Protease in Peptide Boronic Acid Inhibitor Complexes†

William W. Bachovchin,*,‡ Winona Y. L. Wong,‡ Shauna Farr-Jones,‡ Ashok B. Shenvi,§ and Charles A. Kettner§

Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111, and Central Research and Development Department, E. I. du Pont de Nemours and Company, Experimental Station, Wilmington, Delaware 19898

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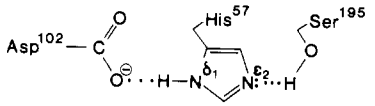
ABSTRACT: ^{15}N NMR spectroscopy was used to examine the active-site histidyl residue of α-lytic protease in peptide boronic acid inhibitor complexes. Two distinct types of complexes were observed: (1) Boronic acids that are analogues of substrates form complexes in which the active-site imidazole ring is protonated and both imidazole N–H protons are strongly hydrogen bonded. With the better inhibitors of the class this arrangement is stable over the pH range 4.0–10.5. The results are consistent with a putative tetrahedral intermediate like complex involving a negatively charged, tetrahedral boron atom covalently bonded to O^{γ} of the active-site serine. (2) Boronic acids that are not substrate analogues form complexes in which $N^{\epsilon 2}$ of the active-site histidine is covalently bonded to the boron atom of the inhibitor. The proton bound to $N^{\delta 1}$ of the histidine in these histidine–boronate adducts remains strongly hydrogen bonded, presumably to the active-site aspartate. Benzeneboronic acid, which falls in this category, forms an adduct with histidine. In both types of complexes the N–H protons of His-57 exchange unusually slowly as evidenced by the room temperature visibility of the low-field ^1H resonances and the ^{15}N–H spin couplings. These results, coupled with the kinetic data of the preceding paper [Kettner, C. A., Bone, R., Agard, D. A., & Bachovchin, W. W. (1988) *Biochemistry* (preceding paper in this issue)], indicate that occupancy of the specificity subsites may be required to fully form the transition-state binding site. The significance of these findings for understanding inhibitor binding and the catalytic mechanism of serine proteases is discussed.

The serine proteases comprise a large and functionally diverse group of proteolytic enzymes. The one feature that these enzymes have in common is that their active sites all contain the same array of aspartic acid, histidine, and serine residues known as the catalytic triad, or charge-relay system[1] (**1**).



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Thus, it is generally assumed that they share a common mechanism for the hydrolytic cleavage of peptide bonds. This mechanism has been the subject of intensive inquiry, making the serine proteases prominent in studies of the molecular basis of enzyme catalytic power and specificity.

Recently, serine proteases have also become prominent as targets of therapeutic agents. For example, derangements in the regulation of leukocyte elastase function are implicated in the pathogenesis of emphysema (Mittman, 1972; Turino et al., 1974; Hance & Crystal, 1975), and the inhibition of this serine protease by a peptide boronic acid inhibitor is effective in preventing elastase-induced emphysema in hamsters (Soskel et al., 1986).

Boronic acids are of considerable interest as inhibitors of serine proteases. Simple alkyl- and arylboronic acids were at first thought to inhibit chymotrypsin and subtilisin by forming adducts with His-57 (Philipp & Bender, 1971; Antonov et al., 1970). However, a consensus was soon reached, supported by the X-ray diffraction studies of subtilisin complexes with benzeneboronic acid and phenylethaneboronic acid, that these inhibitors form tetrahedral adducts with Ser-195 and are, therefore, transition-state-analogue inhibitors (Koehler & Lienhard, 1971; Lindquist & Terry, 1974; Rawn & Lienhard, 1974; Philipp & Maripuri, 1981). This conclusion suggested that changing the R group of the boronic acid to satisfy individual specificity requirements should result in inhibitors that are both potent and specific. This in turn led to the synthesis of peptides containing the boronyl group in place of the C-terminal carboxylate (Matteson et al., 1981; Kettner & Shenvi, 1984). Such peptide boronic acids have been shown to be very effective competitive inhibitors of a number of serine proteases and, indeed, are among the most potent competitive inhibitors of enzymes known. Clearly, a molecular-level understanding of how these inhibitors interact with serine proteases is of considerable interest to both mechanistic enzymology and pharmacology.

These inhibitors, have two curious properties. *First*, they sometimes show what is known as "slow-binding inhibition" (Kettner & Shenvi, 1984). This property seems to correlate with whether or not the inhibitor is a substrate analogue of the target enzyme. Substrate-analogue inhibitors tend to exhibit this property, while non-substrate-analogue inhibitors

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* Author to whom correspondence should be addressed.

‡ Tufts University School of Medicine.

§ E. I. du Pont de Nemours and Co.

[1] The chymotrypsinogen numbering system is used in specifying the residues of the catalytic triad in α-lytic protease and in other serine proteases.

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tend not to exhibit this property (Kettner & Shenvi, 1984; Kettner et al., 1988). *Second*, non-substrate-analogue peptide boronic acid inhibitors exhibit surprisingly high affinities for serine proteases, having K_i values in the 0.1 μM range.

In the present work we have employed ^{15}N NMR spectroscopy to examine His-57 of α-lytic protease in a series of peptide boronic acids complexes. α-Lytic protease is a microbial serine protease that shows sequence and tertiary structural homology with elastase. It also has similar substrate specificity; thus, peptide boronic acids designed to inhibit elastase show similar inhibitory behavior toward α-lytic protease (Kettner et al., 1988). Previous ^{15}N NMR studies of His-57 in α-lytic protease (Bachovchin & Roberts, 1978; Bachovchin, 1986) have demonstrated that the ^{15}N chemical shifts can unambiguously identify the protonation state, the tautomeric structure, and the hydrogen-bonding interactions of the imidazole ring (Schuster & Roberts, 1979; Roberts et al., 1982; Blomberg et al., 1977). This in turn provides information about the immediate environment of His-57. Because of its key location within the active site, this information about His-57 in peptide boronic acid complexes should provide insight into how these inhibitors interact with this enzyme.

A recent ^{15}N NMR study of transition-state-analogue complexes of α-lytic protease led to the proposal that the Asp–His hydrogen bond breaks on forming the transition state during catalysis (Bachovchin, 1986), thereby allowing His-57 to move and perform a dual catalytic role. Because boronic acids are widely believed to be good transition-state analogues, we expected the results of this study to also have significance for this proposal.

Experimental Procedures

Histidine specifically labeled with ^{15}N at $N^{\delta 1}$ (99%) was obtained from ICON (Summit, NJ). DL-Histidine ^{15}N labeled at both ring nitrogens (99%, $N^{\epsilon 2}$ and $N^{\delta 1}$) was obtained from ICON or synthesized by the method of Totter and Darby (Totter & Darby, 1944; Darby et al., 1942). The NIH Stable Isotope Resource of the Los Alamos National Laboratory provided $^{15}NH_3$ (99%) for this synthesis. The purity and ^{15}N content of the labeled histidines were confirmed by ^{15}N and ^1H NMR spectroscopy. The peptide boronic acids were prepared as the pinacol esters or as the free acids by the methods previously described (Kettner & Shenvi, 1984; Kettner et al., 1988). Ac-L-Ala-L-Pro-L-Ala-*p*-nitroanilide was synthesized as previously described (Hunkapiller et al., 1976).

Incorporation of ^{15}N-labeled histidine into α-lytic protease (EC 3.4.21.12) was accomplished by culturing a histidine-requiring mutant of *Lysobacter enzymogenes* (ATCC 29487) as described previously (Bachovchin & Roberts, 1978). Enzyme activity was measured spectrophotometrically at 410 nm ($\Delta\epsilon_{410} = 8.86 \times 10^3$ M^{-1} s^{-1}) with Ac-L-Ala-L-Pro-L-Ala-*p*-nitroanilide (4×10^{-4} M in 0.05 M Tris buffer, pH 8.75 at 25 °C). On the basis of $A_{280}^{1\%} = 8.9$ (Whitaker, 1970), purified preparations of α-lytic protease used in these NMR studies exhibited k_{cat}/K_m values of 2.0×10^3 M^{-1} s^{-1}.

NMR samples were prepared by dissolving lyophilized powders of α-lytic protease in 0.1 M KCl. About 10% ^2H_2O was added to provide an internal lock signal. A small amount of Tris buffer was added (0.05 M) to help stabilize the pH at high pH values. The pinacol esters of the peptide boronic acids were predissolved in a small volume of 0.1 M phosphate buffer, pH 7.0, and incubated for 15–20 min to remove the pinacol protecting group. Such activated peptide boronic acids were then added to the 1–2 mM solutions of α-lytic protease to give a total sample volume of ~2.0 mL. In general, about a 2-fold molar excess of inhibitor over enzyme was employed to ensure full inhibition of the enzyme. The activity of α-lytic protease decreased to near zero within a few minutes following the addition of the inhibitor for all of the peptide boronic acid inhibitors reported here.

^{15}N NMR spectra were recorded at 40.55 MHz on a Bruker AM-400 wide-bore NMR spectrometer equipped with an Aspect 2000A computer and a 10-mm single-frequency ^{15}N probe. Spectra were acquired with a 90° pulse (24 μs), a spectral width of 10000 Hz, 8K real data points, and a recycle time of 0.8 s. Chemical shifts are referenced relative to external 1 M HNO_3 in ^2H_2O with positive shifts being upfield. ^{15}N NMR spectra at low temperatures (218 K) of the imidazole–benzeneboronic acid complex were obtained by using the Bruker variable-temperature accessory. An external capillary containing acetone-d_6 was used to provide the deuterium lock signal for the samples in methanol solution.

^1H NMR spectra were recorded at 400 MHz on the above-described Bruker AM 400 spectrometer with a 5-mm single-frequency ^1H probe. The low-field resonances for the peptide boronic acid–enzyme complexes in general were resolved at room temperature (298 K) in ^1H_2O solutions by using the "2–1–4" pulse sequence for H_2O suppression (Redfield et al., 1975). For the samples examined at low temperature (278 K), the Bruker variable-temperature accessory was employed. Spectra were acquired with a spectral width of 10000 Hz, 8K real data points, and a recycle time of 1.0 s. Chemical shifts are referenced relative to sodium dimethylsilapentanesulfonate (DSS).

The pH of the NMR samples was varied by addition of 0.25 M NaOH or HCl. Enzyme activities and pH values were checked before and after recording each spectrum. In general, the pH measurements agreed to within 0.05 pH unit while those for enzyme activity agreed to within 5%.

Results

^{15}N NMR Spectroscopy. Figure 1 shows ^{15}N NMR spectra of doubly labeled α-lytic protease complexed with Boc-Ala-Pro-boroVal-OH, the most effective peptide boronic acid inhibitor of α-lytic protease examined thus far (K_i of 3×10^{-10} M; Kettner et al., 1988). The proton-decoupled ^{15}N spectra (Figure 1A,C) exhibit two resonances of about equal intensities while the corresponding proton-coupled spectra (Figure 1B,D) exhibit a three-line, tripletlike pattern. These signals occur at relatively high field (~191–194 ppm from external 1 M HNO_3), and their positions do not change significantly with pH over the range 4.0 to 10.5. A comparison of the peak positions in the proton-decoupled and proton-coupled spectra suggests that the tripletlike pattern in the proton-coupled spectra arises from overlapping ^{15}N–H doublets with $^1J_{N-H}$ ~90 Hz. Consistent with this interpretation is the fact that scalar couplings of 90–100 Hz are typical for one-bond ^{15}N–H spin–spin splitting and that ^{15}N chemical shifts of ~191–194 ppm are within the known range for imidazole ring nitrogens with a directly bonded proton.

Complexes of doubly labeled α-lytic protease with MeO-Suc-Ala-Ala-Pro-boroVal-OH,[2] MeOSuc-Ala-Ala-Pro-boro-Ala-OH, Ac-Pro-boroVal-OH give ^{15}N NMR spectra very similar to those with Boc-Ala-Pro-boroVal-OH, i.e., two pH-independent resonances of about equal intensity and with chemical shifts in the region of ~191–194 ppm in proton-

[2] Abbreviations: MeOSuc, methoxysuccinyl; Boc, *tert*-butyloxycarbonyl; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; DIFP, diisopropyl fluorophosphate; TI, tetrahedral intermediate. The prefix boro indicates that the carboxylate of the amino acid residue is replaced by $-B(OH)_2$.

Table I: NMR Parameters of His-57 of α-Lytic Protease in Boronic Acid and Peptide Boronic Acid Inhibited Complexes

inhibitor	^{15}N chemical shifts[a] high pH (~9.0) $N^{\delta 1}$	high pH (~9.0) $N^{\epsilon 2}$	low pH (~4.0) $N^{\delta 1}$	low pH (~4.0) $N^{\epsilon 2}$	^{1}H chemical shifts[a] high pH (~9.0) $N^{\delta 1}$–H	high pH (~9.0) $N^{\epsilon 2}$–H	low pH (~4.0) $N^{\delta 1}$–H	low pH (~4.0) $N^{\epsilon 2}$–H
Type 1 Complexes								
Boc-Ala-Pro-boroVal-OH	194.22	191.45	194.1	190.7	16.00	16.46	15.95	16.40
MeOSuc-Ala-Ala-Pro-boroVal-OH	193.8	191.1	193.6	191.3	16.00	16.53	15.37	16.16
MeOSuc-Ala-Ala-Pro-D,L-boroAla-OH	193.86	192.76	194.0	192.2	16.03	16.44	16.02	16.45
Ac-Pro-boroVal-OH	194.1	192.0	194.0	192.3	16.06	16.46	16.05	16.32
Type 2 Complexes								
MeOSuc-Ala-Ala-Pro-D,L-boroPhe-OH	198.0	166.7	198.0	166.7	15.31	*c*	15.31	*c*
Boc-Ala-Pro-D-boroVal-OH	197.2	168.0	*b*		15.5	*c*	*b*	
benzeneboronic acid	197.5	166.8	*b*		15.2	*c*	*b*	

[a] ^{15}N chemical shifts are in ppm upfield from external 1 M HNO_3 in D_2O; ^{1}H chemical shifts are in ppm downfield from DSS. [b] Inhibitor dissociates under these conditions. [c] Does not apply because in type 2 complexes $N^{\epsilon 2}$ does not have an attached proton.

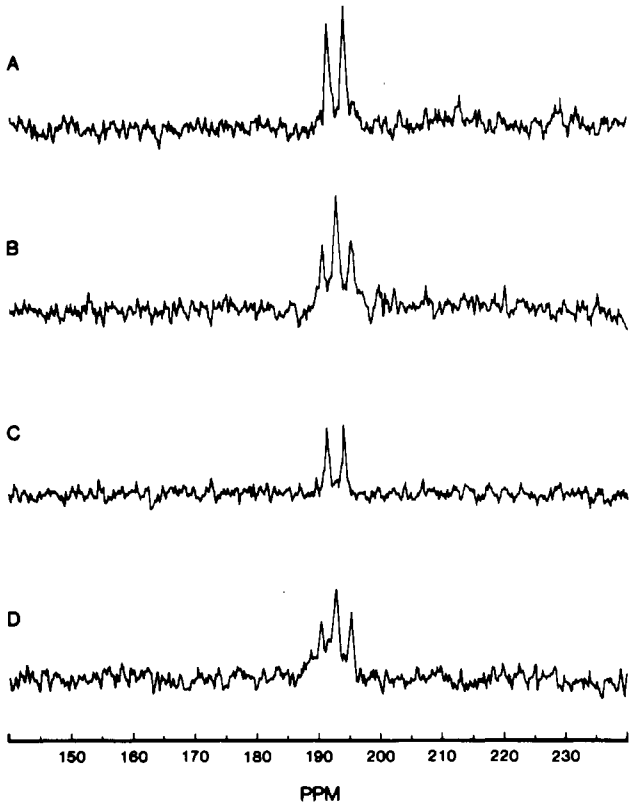


FIGURE 1: ^{15}N NMR spectra (40.55 MHz) of ^{15}N-labeled His-57 α-lytic protease (99% ^{15}N, both ring nitrogens) inhibited with Boc-Ala-Pro-boroVal-OH. (A) pH 5.4, proton decoupled; (B) pH 5.4, proton coupled; (C) pH 9.0, proton decoupled; (D) pH 9.0, proton coupled. The enzyme concentration was about 1 mM. Spectra A, B, and D represent about 58 000 scans, while spectrum C represents about 25 000 scans.

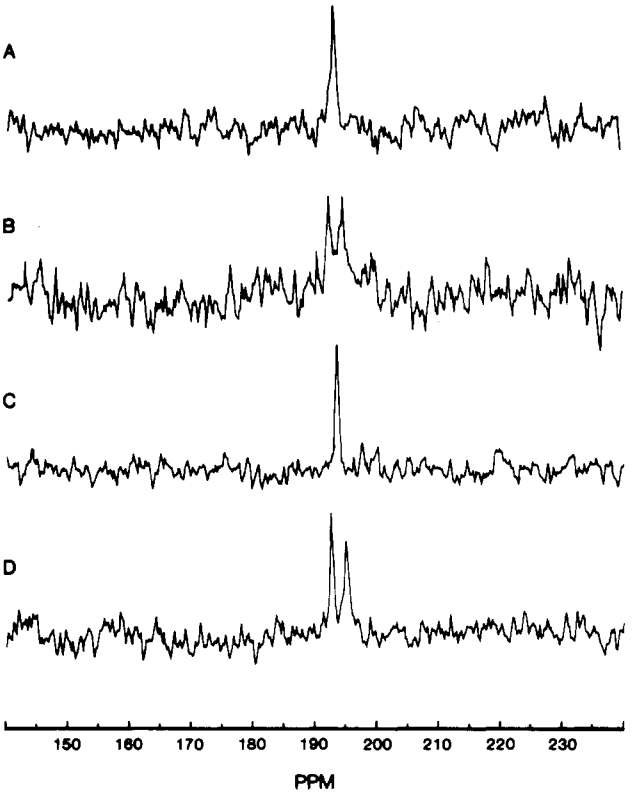


FIGURE 2: ^{15}N NMR spectra (40.55 MHz) of singly labeled α-lytic protease (99% ^{15}N at $N^{\delta 1}$ of His-57) inhibited with MeOSuc-Ala-Ala-Pro-boroVal-OH. (A) pH 4.0, proton decoupled; (B) pH 4.0, proton coupled; (C) pH 9.0, proton decoupled; (D) pH 9.0, proton coupled. Enzyme concentration was about 1 mM, and each spectrum represents about 50 000 scans.

decoupled spectra, which become a tripletlike pattern in proton-coupled spectra. We shall refer to these complexes as type 1 complexes and to the inhibitors as type 1 inhibitors of α-lytic protease.

Type 1 inhibitor complexes with *singly* labeled α-lytic protease exhibit only a single, pH-independent resonance, which becomes a doublet with $^{1}J_{N-H}$ ~90 Hz in proton-coupled spectra, as illustrated in Figure 2 for the complex with MeOSuc-Ala-Ala-Pro-boroVal-OH. The chemical shift of this single resonance always matches that of the higher field of the two ^{15}N resonances observed with doubly labeled enzyme. Thus, we assign the higher field resonance in double labeled type 1 complexes to $N^{\delta 1}$ and the lower field signal to $N^{\epsilon 2}$ of His-57.

The direct observation of ~90-Hz proton splitting on the $N^{\delta 1}$ signal in ^{15}N spectra of single-labeled enzyme–inhibitor complexes confirms our earlier assignment, based on chemical shift arguments, of the tripletlike pattern seen in proton-coupled spectra of doubly labeled type 1 complexes (viz. to account for the observed tripletlike pattern in the presence of a $N^{\delta 1}$ doublet with $^{1}J_{N-H}$ requires that $N^{\epsilon 2}$ also be a doublet with $^{1}J_{N-H}$ ~90 Hz). That both $N^{\epsilon 2}$ and $N^{\delta 1}$ of His-57 exhibit one-bond proton coupling demonstrates that both nitrogens carry a directly bonded proton in type 1 complexes over the entire pH range examined (4.0–10.5). Moreover, that these couplings are resolved at room temperature shows that these protons are not subject to the rapid exchange usually observed for imidazole N–H protons. Table I lists the ^{15}N chemical shifts for type 1 complexes at pH values where His-57 would normally be fully protonated (4.0), and fully neutral (9.0). The ^{15}N shifts, however, were also determined at several pH values between 4.0 and 9.0, to verify their pH independence in each type 1 complex.

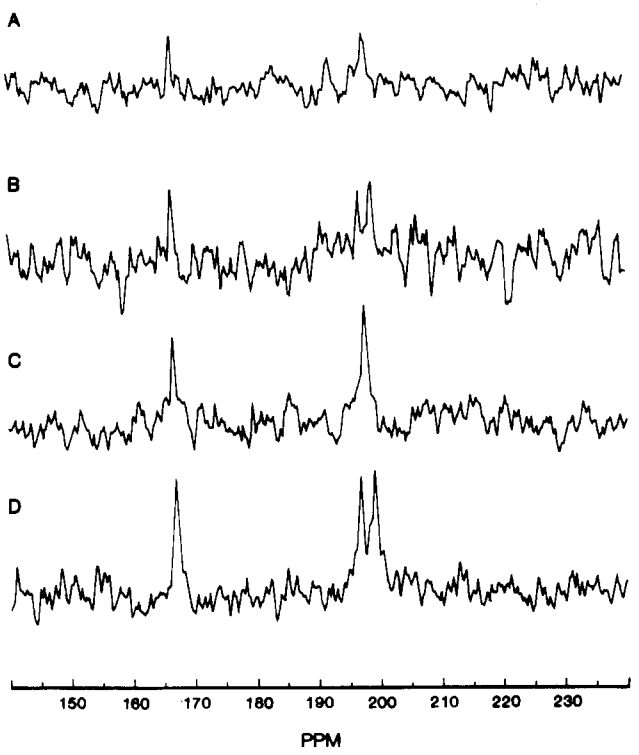


FIGURE 3: ^{15}N NMR spectra (40.55 MHz) of doubly labeled α-lytic protease (99% ^{15}N at $N^{\delta 1}$ and $N^{\epsilon 2}$ of His-57) inhibited with MeO-Suc-Ala-Ala-Pro-boroPhe-OH. (A) pH 4.0, proton decoupled; (B) pH 4.0, proton coupled; (C) pH 9.0, proton decoupled; (d) pH 9.0, proton coupled. Enzyme concentration was about 1 mM. The numbers of scans are 26 000, 67 000, 49 000, and 82 000, for spectra A, B, C, and D, respectively.

Not all peptide boronic acid complexes with α-lytic protease give ^{15}N NMR spectra of the type described above, as Figure 3 illustrates for the complex with MeOSuc-Ala-Ala-Pro-boroPhe-OH. This complex shows one signal at somewhat higher field ($\sim$198 ppm) and a second signal at much lower field ($\sim$166 ppm) than those of type 1 complexes. Neither resonance shows any discernible change in chemical shift over the pH range 4.0–10.5. The signal at $\sim$198 ppm becomes a doublet with $^1J_{N-H}$ $\sim$90 Hz in proton-coupled spectra while the signal at 166 ppm remains a singlet. Complexes of doubly labeled α-lytic protease with Boc-Ala-Pro-D-boroVal-OH or with benzeneboronic acid give ^{15}N spectra virtually identical with those of the MeOSuc-Ala-Ala-Pro-boroPhe-OH complex (Figure 3) but are pH independent only to pH 5.0. At pH 4.0 the spectra revert to that of the native uninhibited enzyme, indicating that between pH 5.0 and 4.0 these inhibitors dissociate from the enzyme. This is consistent, however, with the results of the preceding paper, which show that the affinity of these two inhibitors for α-lytic protease decrease sharply with pH (Kettner et al., 1988). We shall refer to the above complexes as type 2 complexes and to the inhibitors as type 2 inhibitors of α-lytic protease.

With *singly* labeled enzyme, the type 2 inhibitors give ^{15}N NMR spectra showing only a single resonance at high field ($\sim$197–198 ppm) which becomes a doublet in the proton-coupled spectra. On the basis of these results, we assign the high-field signal ($\sim$198 ppm) in the type 2 complexes to $N^{\delta 1}$ and the low-field signal ($\sim$166 ppm) to $N^{\epsilon 2}$ of His-57. The observation of one-bond proton splitting on $N^{\delta 1}$ confirms that this nitrogen carries a proton. That this splitting is resolved at room temperature shows that the $N^{\delta 1}$ proton in type 2 complexes, like the NH protons in the type 1 complexes, exchanges slowly. Table I lists the ^{15}N chemical shifts of the

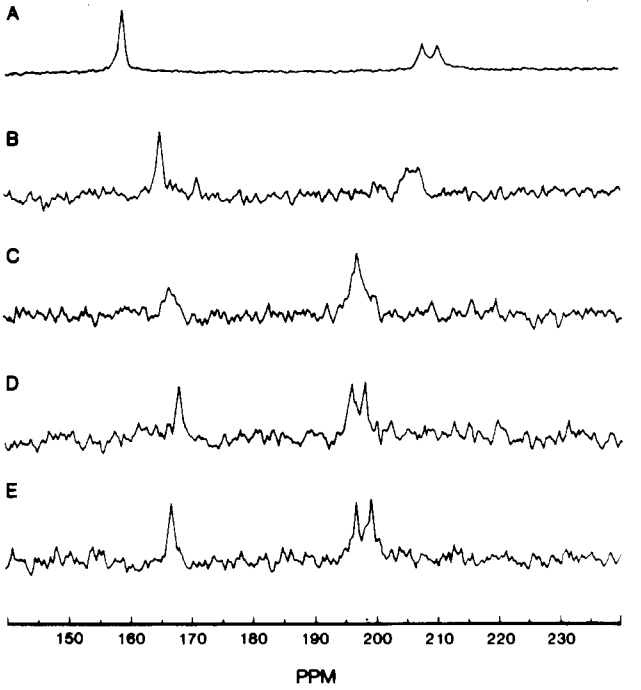


FIGURE 4: ^{15}N NMR spectra (40.55 MHz) of (A) an equimolar mixture of imidazole and benzeneboronic acid in $CHCl_3$ at 218 K, proton coupled; (B) same as (A) but in MeOH; (C) α-lytic protease and benzeneboronic acid, pH 9.0, 278 K, proton decoupled; (D) α-lytic protease and Boc-Ala-Pro-D-Boro-Val, pH 9.0, 298 K, proton coupled; (E) α-lytic protease and MeOSuc-Ala-Ala-Pro-boroPhe-OH, pH 9.0, 298 K, proton coupled. Spectra C–E were obtained with $\sim$1 mM doubly labeled enzyme. Each spectrum represents about 50 000 scans.

type 2 complexes along with those of the type 1 complexes.

Equimolar mixtures of benzeneboronic acid and imidazole in chloroform or methanol at low temperatures (218 K) give ^{15}N NMR spectra quite similar to those observed for type 2 complexes of α-lytic protease, as illustrated in Figure 4. In methanol at 218 K (Figure 4, spectrum B), the ^{15}N chemical shifts are 165.3 and 206.2 ppm. In chlorofom (spectrum A), the ^{15}N shifts are 160 and 209.0 ppm. In both cases the higher field signal shows one-bond ^1H spin coupling in proton-coupled spectra, while the lower field signal does not.

^1H NMR Spectroscopy. Figure 5 illustrates and compares the results of low-field ^1H NMR studies of the type 1 and type 2 boronic acid complexes with α-lytic protease. Type 1 complexes all show two low-field ^1H resonances which, in spectra of doubly labeled α-lytic protease, appear as well-resolved doublets with $^1J_{N-H}$ $\sim$90 Hz as illustrated in Figure 5D for the type 1 complex with Boc-Ala-Pro-Ala-boroVal-OH. The observation of one-bond ^{15}N splitting on both low-field proton signals demonstrates that both signals arise from protons covalently attached to His-57. The chemical shifts of these two ^1H resonances, like the ^{15}N resonances, do not change significantly with pH over the range 4.0–10. With *singly* labeled enzyme, the more downfield doublet collapses to a singlet in all type 1 complexes (Figure 5C), showing that this signal arises from the proton on $N^{\epsilon 2}$ of His-57. Selective ^{15}N decoupling and experiments with unlabeled enzyme confirm the assignment of the lower field ^1H resonance in type 1 complexes to $N^{\epsilon 2}$–*H* and the higher field signal to $N^{\delta 1}$–*H* at all pH values.

Type 2 complexes give low-field ^1H NMR spectra that are quite different from those of type 1 complexes as illustrated in Figure 5A for the MeOSuc-Ala-Ala-Pro-boroPhe-OH complex and in Figure 5B for the Boc-Ala-Pro-D-boroVal-OH complex. Instead of the two ^1H signals in the very low field region of the spectrum ($\sim$16.5 and 16.0 ppm at pH 9.0), type 2 complexes show a single pH-independent resonance some-

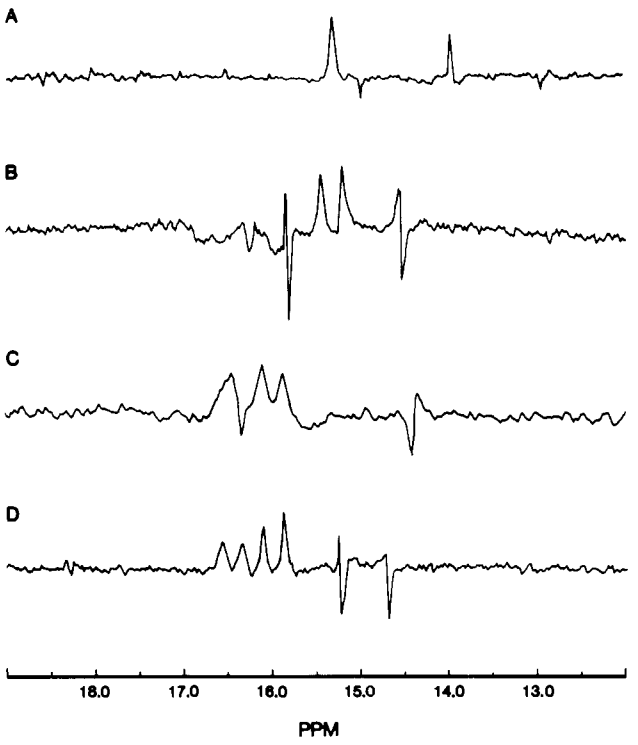
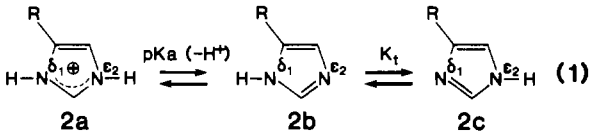


FIGURE 5: Low-field proton NMR spectra of type 1 (C and D) and type 2 (A and B) complexes. (A) Natural abundance α-lytic protease and MeOSuc-Ala-Ala-Pro-boroPhe-OH, pH 9.0; (B) $^{15}N^{\delta 1}$-labeled His-57 α-lytic protease and Boc-Ala-Pro-D-boroVal-OH, pH 9.0; (C) $^{15}N^{\delta 1}$ labeled His-57 α-lytic protease and Boc-Ala-Pro-boroVal-OH, pH 9.0; (D) $^{15}N^{\delta 1}$- and $N^{\epsilon 2}$-labeled His-57 α-lytic protease and Boc-Ala-Pro-boroVal-OH, pH 9.0. Enzyme concentrations were about 1 mM; ^{1}H spectra were acquired by using Redfield (2–1–4) solvent suppression each spectrum represents 1000–2000 scans.

what more upfield (~15.5 ppm). This signal is observable over the pH range 4.0–10.0 in the MeOSuc-Ala-Ala-Pro-boroPhe-OH complex, but only from pH 5.0 to 10.0 in the Boc-Ala-Pro-D-boroVal-OH and benzeneboronic acid complexes. These results coincide with the ^{15}N results in indicating a sharp decrease in affinity with pH for the latter two inhibitors. With *singly* labeled enzyme, the single low-field ^{1}H signal shows ^{15}N spin splitting ($^1J_{N-H}$ ~90 Hz), showing that it arises from a proton bonded to $N^{\delta 1}$ of His-57.

Discussion

Interpretaton of ^{15}N Chemical Shifts. The imidazole ring of histidine can readily assume three structural forms as shown in eq 1. Structure **2a** is the protonated imidazolium ion, and



(1)

2b and **2c** are tautomeric forms of the neutral species. From the standpoint of ^{15}N chemical shifts, the nitrogen atoms within these structural forms can be divided into three bonding states or types (Witanowski et al., 1972; Blomberg et al., 1977; Bachovchin, 1986), which we shall refer to here as NH, =N—, and NH+. NH and =N— refer to the protonated and nonprotonated nitrogens, respectively, within a neutral imidazole ring while NH+ refers to a protonated nitrogen within a protonated and thus positively charged imidazole ring. These nitrogens have previously been referred to as type α, type β, and type α+, respectively (Witanowski et al., 1972; Blomberg et al., 1977; Bachovchin, 1986). Typical ^{15}N chemical shifts for these nitrogen types are given for samples in aqueous solution:

nitrogen type	^{15}N chemical shift (ppm)
NH (type α)	210
=N— (type β)	128
NH+ (type α+)	201

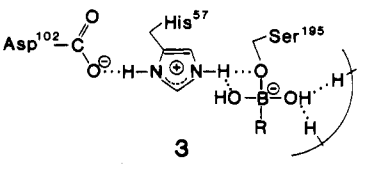
Note that the ^{15}N chemical shifts are quite sensitive to the presence or absence of a proton, there being ~82 ppm difference between NH and =N— nitrogens and ~72 ppm difference between NH+ and =N— nitrogens. The difference between NH and NH+ nitrogens is much smaller but nevertheless real and significant: For a given imidazole nitrogen, the NH+ state invariably resonates 8–10 ppm downfield from the corresponding NH state.

The ^{15}N NMR chemical shifts are also quite sensitive to hydrogen-bonding interactions, undergoing changes that are similar in direction to those induced by protonation or deprotonation but are much smaller. Thus, the resonance of a NH or NH+ nitrogen will move *downfield* up to 10 ppm on becoming a hydrogen-bond donor while a =N— nitrogen will move *upfield* up to 10 ppm on becoming a hydrogen-bond acceptor.

Type 1 Complexes. The ^{15}N chemical shifts and ^{1}H splitting patterns demonstrate that in type 1 complexes both imidazole ring nitrogens of His-57 carry a directly bonded proton and therefore that the imidazole ring is protonated and positively charged even in solutions of pH 10.0. Both imidazole ring nitrogens must therefore be NH+ nitrogens. However, $N^{\delta 1}$ resonates at about 194 ppm and $N^{\epsilon 2}$ at about 191.5 ppm in the four type 1 complexes examined (Table I). These ^{15}N shifts, 8–10 ppm *downfield* from that expected for NH+ nitrogens, indicate that both nitrogens are engaged in strong hydrogen bonds as the donor atoms. [For comparison, in the native enzyme, $N^{\delta 1}$ of His-57 resonates at 191.6 ppm when His-57 is protonated. This ^{15}N shift, 8–10 ppm downfield from NH+ nitrogen, indicates the presence of the hydrogen bond to Asp-102 (Bachovchin & Roberts, 1978; Bachovchin, 1986).]

To test this conclusion, we examined the low-field region of the ^{1}H NMR spectrum of type 1 complexes. Serine proteases in the native state generally show a single low-field ^{1}H resonance, i.e., 14–18 ppm from DSS, which arises from the proton on $N^{\delta 1}$ of His-57 and H bonded to Asp-102. With α-lytic protease this resonance occurs at 13.9 ppm when the imidazole ring is neutral, and at 17.0 ppm when the ring is protonated (Robillard & Shulman, 1974a; Bachovchin, 1985). The H-bonding interaction with Asp-102 is responsible for the abnormally low field chemical shift of this N–H proton and for its exchange rate with H_2O being slow enough to allow its direct observation in ^{1}H NMR spectra. Therefore, if our interpretation of the ^{15}N shifts is correct, we should see not one but two low-field ^{1}H signals in type 1 complexes. Spectra C and D of Figure 5 demonstrate that this is indeed the case. All type 1 complexes (Table I) show two low-field ^{1}H signals, one at about 16.5 ppm and the other at 16.0 ppm. In every case the more downfield signal (i.e., 16.5 ppm) arises from the proton on $N^{\epsilon 2}$ of His-57.

The above results indicate that type 1 complexes have structures as shown in **3**. Note that **3** shows $N^{\delta 1}$ of His-57



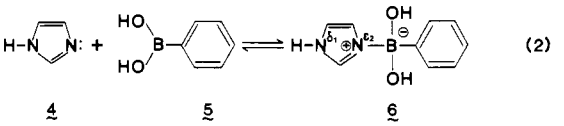
H bonded to Asp-102 and $N^{\epsilon 2}$ H bonded to Ser-195 or to the

boronic acid oxygen, or to both. Although our results do not provide direct information about Ser-195–boronyl group interaction, they support the existence of a tetrahedral and negatively charged boron adduct as shown, because such a negatively charged adduct would tend to stabilize a positively charged His-57 and therefore explain our finding that His-57 remains protonated at pH values as high as 10.5. In concurrent studies, Bone et al. (1987) have also concluded that such a serine–boron adduct is present in a crystalline α-lytic protease–Boc-Ala-Pro-boroVal-OH complex.

Type 2 Complexes. The ^{15}N chemical shift of ~166 ppm, about midway between those characteristic of protonated (NH and NH+) and nonprotonated (=N—) nitrogens, for $N^{\epsilon 2}$ of His-57 in type 2 complexes was at first quite puzzling. It cannot reflect a weighted average of rapidly exchanging NH and =N— nitrogen states because any such mechanism would require that the chemical shift of $N^{\epsilon 2}$ be pH dependent, whereas the ^{15}N shift of $N^{\epsilon 2}$ in type 2 complexes is entirely pH independent. Robillard and Shulman (1974b) have also observed such pH-independent, midrange chemical shift values for the Asp–His H-bonded proton resonance in complexes of chymotrypsin and subtilisin with borate, benzeneboronic acid, and phenylethaneboronic acid. Their interpretation invoked partially protonated states for the imidazole ring, in contrast to a normal imidazole which is either protonated or not. In this model a proton is held in a hydrogen bond (with a single minimum potential energy well) about midway between $N^{\epsilon 2}$ of His-57 and the boronate oxygen atoms. However, such a strongly hydrogen-bonded proton to $N^{\epsilon 2}$ of His-57 should give rise to a low-field ^{1}H resonance or show ^{15}N–H spin coupling. Neither we nor Robillard and Shulman have observed this resonance, and $N^{\epsilon 2}$ of His-57 does not exhibit ^{1}H spin coupling. Thus, a partially protonated imidazole ring does not fit all the evidence.

What then is then explanation for the ^{15}N chemical shift of ~166 ppm for $N^{\epsilon 2}$? Could it reflect a covalent bond between $N^{\epsilon 2}$ of His-57 and the boron? Early studies of alkyl- and arylboronic acids as inhibitors of chymotrypsin and subtilisin were in fact interpreted as indicating the formation of such His-57–boron adducts, although later studies were interpreted in terms of tetrahedral adducts with the active-site serines.

To evaluate this possibility, we investigated the ^{15}N chemical shift behavior of the model complex **6** formed between imidazole (**4**) and benzenboronic acid (**5**). Equimolar mixtures of these compounds form the tetrahedral adduct **6** in water, methanol, or chloroform solution (Phillip & Bender, 1970; North & Wrackmeyer, 1978), with the equilibrium (eq 2)



(2)

4 5 6

favoring the adduct. (We have designated the N–H and N–B nitrogens in the complex **6** as $N^{\delta 1}$ and $N^{\epsilon 2}$ to show parallelism with histidine.)

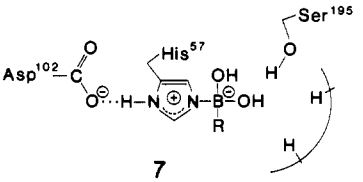
Imidazole in the above solvents at room temperature exhibits a single ^{15}N resonance at (~170 ppm). Only one signal is observed because the N–H proton exchanges rapidly between the two ring nitrogens. The observed ^{15}N shift of **4** (~170 ppm) represents the average of the NH (~210 ppm) and the =N— (~128 ppm) chemical shifts. The addition of 1 molar equiv of benzeneboronic acid to the imidazole solutions, at room temperature, causes the signal to move from 170 to 183 ppm, signaling formation of the complex **6**. However, because only one ^{15}N resonance is observed, exchange of the N–H and N–B nitrogens of the complex must be rapid enough to average their ^{15}N shifts. The most likely exchange mechanism is through equilibration of **6** with **4** and **5** (eq 2).

Lowering the temperature to 218 K slows the exchange sufficiently to allow direct observation of the individual nitrogens of **6**, as Figure 4 illustrates. The N–B ($N^{\epsilon 2}$) and N–H ($N^{\delta 1}$) nitrogens of **6** come into resonance at 165.3 and 206.3 ppm, respectively, in methanol (spectrum B), and at 160 and 209 ppm, respectively, in chloroform (spectrum A). [The small differences in the ^{15}N shifts of **6** between methanol and chloroform solutions can be attributed to differences in hydrogen-bonding abilities of these solvents (Shuster & Roberts, 1979).] In both methanol and chloroform the ^{15}N signal at higher field, assigned to N–H ($N^{\delta 1}$) of **6**, becomes a doublet in ^{1}H-coupled spectra ($^1J_{N-H}$ ~100Hz), while the ^{15}N signal at lower field, assigned to N–B ($N^{\epsilon 2}$) of **6**, remains a singlet. The observation of N–H spin coupling on $N^{\delta 1}$ confirms the following: (i) slow exchange with respect to **6** has been attained, (ii) imidazole retains the $N^{\delta 1}$ proton on forming the complex with benzeneboronic acid, and (iii) the assignments of the ^{15}N signals of **6** are correct.

These results demonstrate that type 2 complexes of α-lytic protease do indeed involve formation of an adduct between $N^{\delta 2}$ of His-57 and the boron atom of the inhibitor. Such a complex accounts for the otherwise difficult to explain ^{15}N shift of ~166 ppm for $N^{\epsilon 2}$ of His-57. It also accounts for the pH independence of both nitrogen signals and for the ^{1}H splitting on $N^{\delta 1}$, but not $N^{\epsilon 2}$ of His-57. The absence of observable ^{15}N–B spin coupling does not weaken this interpretation because such coupling would be too small to resolve in these samples.

The ^{15}N chemical shift of ~198 ppm for $N^{\delta 1}$ of His-57 in type 2 complexes, 8 ppm downfield from the value of 206 ppm observed for $N^{\delta 1}$ of **6**, indicates that it is engaged in a hydrogen bond. To test this conclusion, we examined the low-field ^{1}H NMR spectra of type 2 complexes. If the interpretation of the ^{15}N shifts is correct, these spectra should show one pH-independent, low-field proton signal, which in complexes with singly labeled ($N^{\delta 1}$ of His-57) enzyme will exhibit ^{15}N spin coupling. Spectra A and B of Figure 5 illustrate that this indeed is the case. All type 2 inhibitor complexes exhibit only a single low-field resonance, and in all cases, it has the expected properties. Interestingly, the low-field proton chemical shifts in type 2 complexes, i.e., 15.2–15.5 ppm (Table I), are about midway between the low- (His-57-protonated) and high-pH (His-57-neutral) positions of this Asp–His H-bonded proton resonance in the native enzyme, reminiscent of the $N^{\delta 1}$ and $N^{\epsilon 2}$ nitrogen-15 shifts in the these complexes. Apparently, boronylation has about half the inductive or electronic effect of protonation on the imidazole ring system.

The above results demonstrate that type 2 complexes of α-lytic protease involve formation of an adduct between $N^{\epsilon 2}$ of His-57 and boron of boronic acid inhibitors while $N^{\delta 1}$ of His-57 remains hydrogen bonded, presumably to Asp-102 (see structure **7**). Is it possible that type 2 complexes also might



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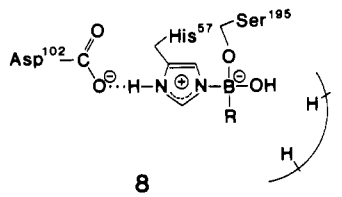
involve esterification of Ser-195 to form the diadduct, bridging structure shown in **8**? Although we favor structure **7**, our



8

present results do not allow us to unequivocally distinguish between structures **7** and **8**. Studies with this objective are currently under way.

NH Proton Exchange Rates. The NH protons of histidyl residues in proteins are normally not observable by ^{1}H NMR because of their exceedingly rapid exchange with solvents protons (Bachovchin & Switzman, 1983). Even the strongly hydrogen bonded N$^{\delta 1}$–H proton of His-57 in serine proteases in the native, resting state exchanges sufficiently rapidly that it cannot be observed at room temperature (Robillard & Shulman, 1974a,b). Enzyme samples must be cooled to about 278 K to make observation possible by ^{1}H NMR. Even then, extreme pH values may also be required (Bachovchin, 1985). Thus, it is remarkable that the NH protons of His-57 in type 1 and type 2 boronic acid complexes and their ^{15}N–H spin couplings are observable at room temperature. One explanation for the slow NH exchange rates is simply that the enzyme-bound inhibitors shield His-57 from contact with solvent. Another is that the proximity of the negatively charged boron atom to His-57 inhibits the normal exchange mechanisms.

Implications for Transition-State Interactions in Inhibition and Catalysis. Serine protease active sites are generally believed to contain a subsite that is complementary to the transition state and a series of specificity subsites that are complementary to the substrate. The effectiveness of boronic acids as inhibitors of these enzymes has been widely attributed to the boronyl group's ability to form a tetrahedral adduct with Ser-195 that mimics the transition state and binds tightly to the transition-state subsite. The increased effectiveness of peptide boronic acids over simple alkyl- and arylboronic acid is attributed to the additional favorable contacts these molecules can make with the specificity subsites.

Our present work indicates that this conventional model of the active site of serine proteases and of the mechanism of boronic acid inhibition is oversimplified, if not incorrect. According to this model the binding affinity of small boronic acid inhibitors such as borate and benzeneboronic acid, which lack the structure to make additional contacts with the enzyme, favorable or unfavorable, should reflect the affinity of the boronate moiety for the transition-state binding site. This work, however, shows that these inhibitors form adducts with His-57, not Ser-195. Considering the high K_i values of these inhibitors, the preferences for adding to His-57 over Ser-195 indicates low intrinsic affinity of the boronate moiety for the putative transition-state binding site.

Ser-195 adducts, however, are formed with peptide boronic acids that are analogues of good substrates, suggesting that the specificity subsites play a role in forming the transition-state-like complexes. Perhaps the favorable contacts between the amino acid residues of type 1 inhibitors and the specificity subsites simply pay for the formation of the energetically less favorable Ser-195 adduct in place of the more favorable His-57 adduct. The observation that the type 2 inhibitors MeO-Suc-Ala-Ala-Pro-boroPhe-OH (K_i = 5.4 × 10^{-7} M) and Boc-Ala-Pro-D-boroVal-OH (K_i = 6.2 × 10^{-7} M) actually have higher affinities for α-lytic protease than the type 1 inhibitor Ac-Pro-boroVal-OH (K_i = 3.3 × 10^{-6} M) lends credence to this supposition.

How then does one explain the extraordinary high affinities, K_i's of 1 × 10^{-10}–1 × 10^{-12} M (C. A. Kettner, unpublished results), of the better peptide boronic acid inhibitors? Such high affinities are observed only with substrate analogues and only when the peptide consists of at least three amino acids. These better inhibitors, therefore, have better prospects for favorable interactions with the enzyme's specificity subsites. But such interactions alone cannot account for the unusually high affinities of these inhibitors because homologous peptides, peptide aldehydes, peptide trifluoromethyl ketones, and other homologous peptide derivatives lacking the boronyl functionality exhibit much lower affinities. The boronyl groups of the better peptide boronic acid inhibitors must therefore contribute substantially to their binding energy. This in turn implies the existence of some type of synergism between the specificity substites and the transition-state site. Perhaps occupancy of the enzyme's specificity subsites is required to form the transition-state binding site. The effect could be such that as the contacts in the specificity sites become increasingly favorable, the transition-state binding site becomes increasingly shaped to bind the transition state, and thus formation of a boron–serine adduct becomes increasingly favorable energetically. The crystal structure of α-lytic protease complex to Boc-Ala-Pro-boroVal-OH does not show any large conformational changes from the native structure. It does show, however, a number of small changes in the positions of side-chain and main-chain atoms in and around the active site, including a 0.26-Å movement of His-57 and a 120° rotation of the methyl group of Met-192. The latter change is particularly relevant because Met-192 forms part of the P_1 binding site, while its sequential neighbor, Gly-193, is involved in forming the oxyanion hole (Bone et al., 1987). Alternatively formation of a Ser-195 adduct, although thermodynamically very favorable, may have a high activation energy barrier. With type 1 inhibitors, a precomplex involving occupancy of the specificity substrates by the inhibitor could serve to hold the boronate group in close proximity to Ser-195 for a time sufficient to allow formation of the adduct. Either of the above mechanisms has the additional benefit of providing a plausible explanation for slow-binding kinetics and for why this property is associated with substrate-analogue inhibitors; i.e., in the first mechanism formation of the transition-state binding site is slow, while in the second, formation of the Ser-195–boron adduct is slow, following the rapid formation of a precomplex involving occupancy of the specificity subsites.

An interplay between the specificity subsites and the transition-state binding site, very similar to that discussed above for boronic acid inhibition, is also frequently observed during catalysis with α-lytic protease and other serine proteases (Thompson, 1973; Fersht, 1984; C. A. Kettner, unpublished results; Stein, 1983, 1985). In these studies, increasing the chain length of peptide substrates results not in lowered K_m or improved binding, as might be expected from the improved prospects for specificity subsite interactions, but rather in increased k_{cat}. These results have often been interpreted as support for the strain theory wherein the enzyme, having a rigid transition-state binding site, uses the substrate specificity site binding energies to distort or strain the substrate toward the transition state. Because the ground-state structure of boronic acids is already complementary to the putative transition-state binding site, the present results suggest the reverse mechanism, i.e., that occupancy of the specificity subsite induces the enzyme to assume a structure complementary to the transition state. The latter mechanism has the advantage of explaining the apparent synergism between the specificity

subsites and the transition-state site in both inhibition and catalysis, whereas the strain theory explains only the catalytic synergism.

Affinity of Type 1 Peptide Boronic Acid Inhibitors. Why are substrate-analogue boronic acids 3–4 orders of magnitude more effective as serine protease inhibitors than the corresponding aldehydes? For example, MeOSuc-Ala-Ala-ProboroVal-OH (K_i = 6.7 × 10^{-9} M; Kettner et al., 1988) binds to α-lytic protease abut (4 × 10^3)-fold better than the corresponding peptide aldehyde (K_i = 2.6 × 10^{-5} M; C. A. Kettner, unpublished results). Because aldehydes are also expected to form tetrahedral adducts with Ser-195 (Shah et al., 1984; Mackenzie et al., 1984; Malthouse, 1986), the large difference must reflect differences in the stereoelectronic properties of their respective adducts.

One difference concerns the chemical nature of the substituent groups to the tetrahedral center. The boronic acid adducts have a hydroxyl group where the aldehyde adducts have a proton; boronic acids can, therefore, form two hydrogen bonds with the enzyme whereas the aldehyde adducts can form only one. That the boronic acid adducts actually form this additional hydrogen bond is supported by our finding that $N^{\epsilon 2}$ of His-57 is strongly hydrogen bonded in type 1 complexes. This extra hydrogen bond may partially explain why boronic acids are more effective inhibitors than aldehydes.

Another difference concerns the location of negative charge. The boronic acid adducts have a negative charge on the boron atom whereas the aldehyde adducts may have a negative charge on the hemiacetal oxygen, although this has not been established. The present work demonstrates that, in complexes with substrate-analogue boronic acids, His-57 remains fully protonated, and thus positively charged, even at pH values as high as 10.5. This is unusual for histidyl residues in proteins and must reflect the presence of strong stabilizing interactions. A hydrogen bond between $N^{\epsilon 2}$ of His-57 and a hydroxyl group of the boronic acid adduct, by itself, cannot account for the stability of the protonated form of His-57 because the hydroxyl group could equally well hydrogen bond with a neutral His-57. Furthermore, His-57 in the resting enzyme is strongly hydrogen bonded at both imidazole ring nitrogen positions, and this does not prevent it from titrating with near-normal pK_a (Bachovchin, 1986). The persistence of the protonated form of His-57, therefore, must be due to the negative charge on the nearby boron atom and to the electrostatic forces generated by the resulting –, +, – configuration of charges on Asp-102, His-57, and the boron atom, respectively. The perturbation in the pK_a of His-57 to above 10.5 indicates a minimum ΔG of 6–8 kcal/mol for this electrostatic interaction. That comparable electrostatic interactions are indeed absent in peptide aldehyde–enzyme complexes is indicated by the available NMR data on His-57 of serine proteases, which show that in these complexes His-57 titrates with a more or less normal pK_a (Hunkapiller et al., 1975; S. Farr-Jones, W. W. Bachovchin, and C. A. Kettner unpublished results). It seems likely, therefore, that the location of a negative charge on boron in the tetrahedral adduct with Ser-195, and the resulting electrostatic interaction with His-57 (and Asp-102), is responsible for much, if not all, of the increased affinity of peptide boronic acids relative to corresponding peptide aldehydes.

Warshel has proposed that electrostatic interactions within enzyme active sites are likely to be surprisingly large (Warshel, 1981). He has argued that they may even represent the single most important factor in enzyme catalysis (Warshel, 1978). Such arguments support the hypothesis that electrostatic interaction between His-57 and boron contributes substantially to the binding affinity of peptide boronic acid inhibitors.

Alkyl- and Arylboronic Acid Inhibitors. Our finding that favorable contacts with the enzyme's specificity subsites are required for boronic acid inhibitors to form adducts with Ser-195 suggests that many previously studied alkyl- and arylboronic acid–serine protease complexes may have been histidine, rather than serine, adducts. Until recently, the only available crystal structures of such complexes were those of subtilisin with benzeneboronic acid and phenylethaneboronic acid (Matthew et al., 1975). In both cases, the X-ray maps have been interpreted as indicating a covalent tetrahedral Ser-195 adduct. More recently, an X-ray structure of dimeric α-chymotrypsin inhibited with phenylethaneboronic acid (Tulinsky & Blevins, 1987) has been reported. This work was also interpreted as indicating a tetrahedral adduct with Ser-195, although only in one molecule of the dimer. Although we find that benzeneboronic acid forms a type 2 complex (histidine adduct) with α-lytic protease, while the X-ray diffraction work indicates that it and phenylethaneboronic acid form type 1 like complexes (Ser-195 adducts) with subtilisin and, under some conditions, with chymotrypsin, these results do not necessarily conflict. Subtilisin and chymotrypsin cleave peptides after phenylalanine, and their S_1 specificity pockets are designed to interact favorably with aromatic rings. Perhaps benzeneboronic acid and phenylethaneboronic acid are able to make sufficiently favorable contacts with the S_1 specificity subsite and with Ser-195 of subtilisin and chymotrypsin, such that they mimic substrate-analogue or type 1 inhibitors of these enzymes.

However, Robillard and Shulman (1974a,b) have conducted low-field ^{1}H NMR studies of these same complexes and reported a single pH-independent resonance with a midrange chemical shift. The present work, having shown that low-field signals with the above-described properties are characteristic of type 2 complexes and are quite uncharacteristic of type 1 complexes, indicates that the above work should now be reinterpreted as evidence for His-57–boronic acid adducts. This interpretation, however, puts the low-field ^{1}H NMR studies in conflict with the X-ray diffraction studies (Matthews et al., 1975). Thus complexes of benzeneboronic acid and phenylethaneboronic acid with subtilisin and chymotrypsin need to be reexamined by both NMR and X-ray diffraction.

Implications for a Moving Histidine Mechanism. Recent ^{15}N NMR studies of His-57 of α-lytic protease have shown that, in the tetrahedral, transition-state-analogue complexes formed with PMSF and DIFP, the Asp–His H bond breaks when His-57 becomes protonated on lowering the pH (Bachovchin, 1986). The structural similarity of the low-pH complexes to the putative tetrahedral intermediate or transition state, formed with real substrates (both contain a tetrahedral Ser-195 adduct and a protonated His-57), prompted the proposal that Asp–His H-bond rupture might also occur as part of the catalytic mechanism where it would permit His-57 to move at a crucial moment. The present work, however, shows that the Asp–His H bond does not break in type 1 peptide boronic acid complexes, even though these complexes also contain a tetrahedral adduct to Ser-195 and a protonated His-57. Do these results rule out a moving histidine mechanism?

The answer depends on how well the type 1 boronic acid complexes model the true transition-state complex. By definition, no analogue complex is an exact model of a true transition-state complex. In particular, the analogue complexes obtained with type 1 peptide boronic acids differ from true

transition-state complexes in having a negative charge on the tetrahedral boron atom. The present work shows that the proximity of this negative charge to His-57 leads to a strong electrostatic interaction with His-57 that keeps it positively charged even at pH values as high as 10.5. Such a strong electrostatic interaction should also tend to prevent a positively charged His-57, otherwise inclined to move, from moving.

The behavior of His-57 in peptide aldehyde complexes should provide a useful test of the above suppositions concerning the effects of negative charge on boron in the type 1 boronic acid adducts. As discussed in some detail earlier, these complexes closely resemble the peptide boronic acid complexes with respect to specificity subsite interactions and to the formation of a tetrahedral and negatively charged adduct with Ser-195. In aldehyde complexes, however, the negative charge is supposedly located on the hemiacetal oxygen, one bond further from His-57 than that of the boronic acids and in a better position to interact favorably with the oxyanion hole. In this respect, the aldehyde complexes are better models of the putative tetrahedral intermediate, which has a negative charge on the carbonyl oxygen of the scissile bond, than are the boronic acids. Preliminary ^{15}N spectra of two different peptide aldehyde (Ac-Ala-Ala-Pro-Ala-al and MeOSuc-Ala-Ala-Pro-Val-al) complexes of α-lytic proteases have been obtained (S. Farr-Jones, C. A. Kettner, and W. W. Bachovchin, unpublished results). In both cases, the ^{15}N shifts show that the Asp–His H bond breaks on protonating His-57 at low pH, just as in the PMSF- and DIFP-inhibited complexes. Further evidence for such Asp–His H-bond rupture in peptide aldehyde complexes of serine proteases comes from an X-ray diffraction study of *Streptomyces griseus* protease A in a complex with a synthetic tetrapeptide aldehyde. This study showed that, at low pH, in the peptide aldehyde complex but not in the corresponding peptide alcohol complex, His-57 undergoes a large conformational movement (Brayer et al., 1979; James et al., 1980). These results support the above supposition about the role of the negative charge on boron in the boronic acid complexes in preventing the Asp–His H-bond rupture and consequent movement of His-57. Whether or not peptide aldehyde complexes are actually better models of the true tetrahedral intermediate than are boronic acid complexes remains a matter for speculation. Nevertheless, until this is resolved, the moving histidine mechanism should not be ruled out.

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