Identification of Serine and Histidine Adducts in Complexes of Trypsin and Trypsinogen with Peptide and Nonpeptide Boronic Acid Inhibitors by ^{1}H NMR Spectroscopy†

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ABSTRACT: We have previously shown, in ^{15}N NMR studies of the enzyme's active site histidine residue, that boronic acid inhibitors can form two distinct types of complexes with α-lytic protease. Inhibitors that are structural analogs of good α-lytic protease substrates form transition-state-like tetrahedral complexes with the active site serine whereas those that are not form complexes in which $N^{\epsilon 2}$ of the active site histidine is covalently bonded to the boron of the inhibitor. This study also demonstrated that the serine and histidine adduct complexes exhibit quite distinctive and characteristic low-field ^{1}H NMR spectra [Bachovchin, W. W., Wong, W. Y. L., Farr-Jones, S., Shenvi, A. B., & Kettner, C. A. (1988) *Biochemistry 27*, 7689–7697]. Here we have used low-field ^{1}H NMR diagnostically for a series of boronic acid inhibitor complexes of trypsin and trypsinogen. The results show that H-D-Val-Leu-boroArg and Ac-Gly-boroArg, analogs of good trypsin substrates, form transition-state-like serine adducts with trypsin, whereas the nonsubstrate analog inhibitors boric acid, methane boronic acid, butane boronic acid, and triethanolamine borate all form histidine adducts, thereby paralleling the previous results obtained with α-lytic protease. However, with trypsinogen, Ac-Gly-boroArg forms predominantly a histidine adduct while H-D-Val-Leu-boroArg forms both histidine and serine adducts, with the histidine adduct predominating below pH 8.0 and the serine adduct predominating above pH 8.0. The addition of exogenous Ile-Val, the dipeptide formed at the amino terminus of trypsin upon activation of trypsinogen to trypsin, induces both the Ac-Gly-boroArg and the H-D-Val-Leu-boroArg trypsinogen complexes to switch from histidine and pH-dependent multiple mode adducts to pH-independent, well-defined serine adducts. All the nonsubstrate analogs listed above that form histidine adducts with trypsin also form histidine adducts with trypsinogen. The significance of these findings for understanding the mechanisms of catalysis, inhibitor binding, and zymogen activation is discussed.

Serine proteases constitute an exceptionally large and functionally diverse class of enzymes. The active sites of these enzymes all contain the same geometrical arrangement of aspartic acid, histidine, and serine known as the catalytic triad. The better known members of this group such as trypsin, chymotrypsin, elastase, subtilisin, and α-lytic protease have been the subjects of intense structural and mechanistic scrutiny, thereby making these enzymes important as model systems for studies of the molecular basis of enzyme catalytic power and specificity. Many other members of this group have been demonstrated to play crucial roles in a number of important biological processes, such as in blood coagulation (Furie & Furie, 1988), fibrinolysis (Collen & Lijnen, 1991), complement activation (Frank & Fries, 1989), bacterial pathogenesis (Plaut, 1983), and T cell activation (Schön et al., 1985). Owing to their crucial roles in these important processes, many of these enzymes are the target of active drug design and development efforts. Moreover, new serine proteases continue to be discovered; thus interest in serine protease structure, mechanism, biological functions, and inhibitor design should continue to grow.

Peptide boronic acids are exceptionally potent inhibitors of serine proteases. Because their potency as inhibitors of serine proteases is widely believed to derive from the boronyl group's ability to mimic the transition state of the enzyme-catalyzed reaction, boronic acids inhibitors are of considerable interest in structural and mechanistic studies of serine proteases as models of true enzyme–transition state complexes. Because their high potency can be combined with high specificity for a target protease through appropriate construction of the peptide portion of the inhibitor (Kettner & Shenvi, 1984), peptide boronic acids are also of considerable interest to drug development efforts and of utility to in vivo biochemical studies of serine proteases biological functions (Flentke et al., 1991).

We have previously demonstrated, in ^{15}N NMR[1] studies of the active site histidine residue (Bachovchin et al., 1988), that boronic acid inhibitors form two distinct types of complexes with α-lytic protease, a bacterial serine protease similar to elastase in sequence, structure, and specificity (Kaplan et al., 1970). Boronic acid derivatives that are analogs of good α-lytic protease substrates form transition-state-like tetrahedral complexes with the active site serine as shown in **1**.[2] In this type of complex, the ^{15}N chemical shifts show that both imidazole ring nitrogens are directly bonded to protons, that both NH groups are strongly hydrogen bonded, and that the

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[1] Abbreviations: NMR, nuclear magnetic resonance; Ac, acetyl; Boc, *tert*-butyloxycarbonyl; BPTI, bovine pancreatic trypsin inhibitor; MeO-Suc, methoxysuccinyl; pGB, *para*-guanidinobenzoyl; PSTI, pancreatic soybean trypsin inhibitor; PTI, pancreatic trypsin inhibitor. The prefix boro indicates that the carboxylate of the amino acid residue is replaced by $-B(OH)_2$.

[2] The chymotrypsinogen numbering system is used in specifying the residues of the catalytic triad in α-lytic protease, trypsin, and trypsinogen.

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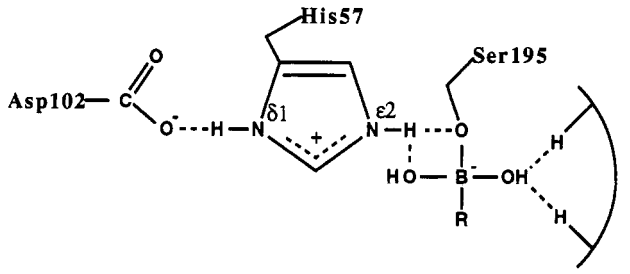

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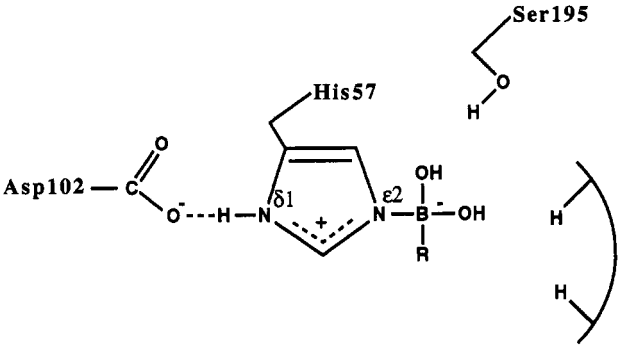
complex, which includes a protonated imidazole ring, is stable to pH over the range 4 to above 10. However, boronic acid derivatives that are not analogs of good substrates, such as MeOSuc-Ala-Ala-Pro-boroPhe, Boc-Ala-Pro-D-boroVal, and benzene boronic acid, form complexes with α-lytic protease in which $N^{\epsilon 2}$ of the imidazole ring is covalently bonded to the boron atom of the inhibitor while $N^{\delta 1}$ remains protonated and hydrogen bonded to Asp102 as shown in **2**. Although the ^{15}N



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NMR results unequivocally demonstrated the existence of the N–B bond, they did not permit us to discriminate between the adduct involving only the histidine as ligand as shown in **2** or a diadduct involving both the histidine and serine residues as ligands.

X-ray crystallography studies have since confirmed the existence of the N–B bond in the complex of α-lytic protease with MeOSuc-Ala-Pro-boroPhe, while further resolving the question above in favor of a His, Ser diadduct, at least for this one particular complex (Bone et al., 1989). The ^{15}N NMR results further demonstrated that the histidine adduct complex **2**, like the serine adduct complex **1**, is stable to pH, although over a slightly smaller pH range.

That α-lytic protease forms histidine adducts with boronic acid inhibitors that have the wrong stereochemistry about the C^{α} carbon of the P_1 residue,[3] such as Boc-Ala-Pro-D-boroVal, or that have the wrong amino acid residue in the P_1 position, as in MeOSuc-Ala-Ala-Pro-boroPhe, is relatively easy to rationalize. Strongly unfavorable interactions (between an incorrectly oriented P_1 Val residue and the enzyme in the first case or between a P_1 Phe residue and an S_1 specificity site designed to recognize Ala or Val in the second) prevent correct binding of the inhibitor, which then interacts with the enzyme in the next most favorable way. However, if the potency of boronic acid as inhibitors derives from the boronyl group being an especially good mimic of the transition state, as is widely believed, it is more difficult to explain why simple boronic acid derivatives, such as boric acid itself, and most especially Ac-boroAla (Farr-Jones et al., unpublished results) form histidine adducts rather than transition-state-like serine adducts with α-lytic protease as these inhibitors do not have structural features to prevent the putatively strong transition-state-like enzyme–boronyl interaction. That they form histidine adducts suggests that the specificity subsites may play a more significant role in transition-state binding than previously supposed. However, this phenomenon, that a serine protease forms transition-state-like serine adducts only with boronic acid inhibitors that are able to make favorable contacts with the specificity subsites and histidine adducts with those that cannot, has thus far only been demonstrated for α-lytic protease. It should be of some interest and importance to establish whether this phenomenon is unique to α-lytic protease or whether it is general, i.e., will all serine proteases exhibit this behavior?

Unfortunately, the incorporation of ^{15}N-labeled histidine into serine proteases other than α-lytic protease is in most instances still either not feasible or practical. However, many serine proteases exhibit a single proton resonance at very low field which arises from the proton in the hydrogen bond between Asp102 and His57 (Robillard & Shulman, 1974a,b; Markley, 1978). For α-lytic protease in the native resting state, this signal occurs at 13.8 ppm when the imidazole ring of His57 is neutral and at 17 ppm when it is protonated. The assignment of this signal to the proton on $N^{\delta 1}$ was made unequivocal by the observation of ^{15}N splitting in selectively [$^{15}N^{\delta 1}$]His57-labeled enzyme (Bachovchin, 1985). The two types of boronic acid inhibitor complexes formed with α-lytic protease described above give quite distinct and characteristic ^1H spectra in this low-field region. Complexes of the type shown in **1** involving a serine adduct exhibit two low-field signals, one at ~16.0 ppm from the proton on $N^{\delta 1}$ on His 57 and a second at ~16.5 ppm from the proton on $N^{\epsilon 2}$. Like the ^{15}N signals, the protons signals are pH independent. In contrast, the low-field region of complexes of the second type, involving a histidine adduct as in **2**, exhibits only a single, pH-independent, resonance at ~15.5 ppm, from the proton on $N^{\delta 1}$.

The above results indicate that it should be possible to use the low-field ^1H spectra diagnostically on boronic acid inhibited complexes of serine proteases that cannot be easily labeled with [^{15}N]histidine. Here we report such studies of trypsin and trypsinogen. Our main objective in undertaking this study was to determine whether or not the phenomenon discovered in α-lytic protease also occurs with trypsin. Trypsin is made as an inactive zymogen, and the zymogen is readily available. X-ray crystallographic studies (Fehlhammer et al., 1977; Kossiakoff et al., 1977; Huber & Bode, 1978) have shown that the active site catalytic triad is fully formed in trypsinogen but that the oxyanion hole and the specificity subsites are not. We therefore expected comparative studies of boronic acid inhibited complexes of trypsin and trypsinogen to yield some interesting insights into the structural and functional properties of serine proteases and into the mechanisms of zymogen activation and catalysis. Finally, trypsin-like enzymes play essential regulatory roles in a number of important processes such as in the blood coagulation, fibrinolytic, and complement systems. Thus, the mechanism of boronic acid inhibition of trypsin is of considerable interest from the standpoint of drug design and development efforts as the information obtained with trypsin should serve as a model for boronic acid interactions with trypsin-like enzymes in general. This paper represents the first structural studies of specific peptide–boroArg complexes of trypsin and trypsinogen.

[3] The nomenclature of Schechter and Berger (1967) is used to designate the individual amino acids (P_3, P_2, P_1) of the peptide substrate.

EXPERIMENTAL PROCEDURES

Porcine pancreatic trypsin (EC 3.4.21.4) (type IX, T-0134), bovine pancreatic trypsinogen (T-1143), and boric acid were purchased from Sigma Chemical Co. (St. Louis, MO). Butane boronic acid, methane boronic acid, and triethanolamine borate were purchased from Aldrich (Milwaukee, WI). The dipeptide Ile-Val was purchased from ICN Biomedicals, Inc. (Irvine, CA). Ac-Gly-boroArg and H-D-Val-Leu-boroArg were synthesized by methods previously described (Kettner et al., 1990).

NMR samples were prepared by dissolving lyophilized enzyme in 400 μL of 0.1 M KCl with about 10% D_2O added to provide the field frequency lock signal. Enzyme concentrations were typically about 2 mM. Inhibitor boronic acids were added directly to the above NMR samples. The amount of inhibitor added was normally sufficient to saturate the enzyme. The amount needed for saturation varied from just a little more than stoichiometric for the tight binding peptide boronic acids, which have K_i values for trypsin in the subnanomolar range at neutral pH values, to 20-fold molar excesses for the more weakly binding alkyl boronic acids. In some cases, saturation was not easily achieved because of the weaker binding of the inhibitors at pH extremes or because of the weaker binding of the inhibitors to trypsinogen compared to trypsin. In these cases, however, the exchange rate of enzyme-bound inhibitor with free inhibitor was slow as demonstrated by the appearance of separate signals in the ^1H NMR spectra from resting and inhibited enzyme. Thus, the ^1H NMR signals assigned to each inhibitor complex, in every case, have chemical shifts directly representing those of the complex unaffected by exchange averaging between free and resting enzyme. Enzyme activity was measured spectrophotometrically at 410 nm using *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide (Sigma Chemical Co.) (Lottenberg et al., 1981). For experiments involving the dipeptide Ile-Val, 0.1 M Ile-Val was added to the trypsinogen–inhibitor complexes (Bode & Huber, 1976). The pH of the NMR samples was varied by addition of 0.25 M NaOH or HCl. The pH of the samples were measured before and after recording each spectrum, and these values agreed to within 0.05 pH units.

^1H NMR spectra were recorded at 400 MHz on a Bruker AM-400 wide-bore NMR spectrometer equipped with an Aspect 3000 computer and a 5-mm single-frequency ^1H probe. The samples were maintained at low temperature (278 K) using the Bruker variable-temperature accessory, and spectra were acquired using the "hard 1–1" pulse sequence (Clore et al., 1983) with oversampling (spectral width of 40 000 Hz, 32K real data points) (Delsuc & Lallemand, 1986). The NMR data were processed on a Sun computer using Dennis Hare's program, Felix 2.0.

RESULTS

Figure 1 shows the low-field ^1H NMR spectra of trypsin complexes with two tight binding peptide boronic acid inhibitors, H-D-Val-Leu-boroArg (spectra A and B) and Ac-Gly-boroArg (spectra C and D). Both inhibitors are "slow" tight binding inhibitors of trypsin. Ac-Gly-boroArg has a K_i (initial) of 3.4 nM ± 1.4 and a K_i (final) of 0.64 nM ± 0.05, while H-D-Val-Leu-boroArg is even a more potent inhibitor with a K_i (initial) of 5.5 nM ± 1.6 and a K_i (final) of 0.21 nM ± 0.09. Both inhibitors give complexes with trypsin which exhibit two pH-independent signals (compare spectrum A with spectrum B and C with D) at ~16.3 and 17 ppm, with about equal intensity and line width (~80 Hz). The complexes are stable over the pH range of 3.5–10 (Table I). For comparison, uninhibited trypsin exhibits one pH-dependent, low-field signal which moves from 14.5 ppm at high pH (spectrum E) to 17.6 ppm at low pH (spectrum F) as the active site imidazole ring titrates. The behavior of this signal in trypsin has been previously reported (Markley, 1978). The spectra of the two peptide boronic acid–trypsin complexes are strikingly similar to those exhibited by Boc-Ala-Pro-boroVal and other specific peptide boronic acid complexes of α-lytic

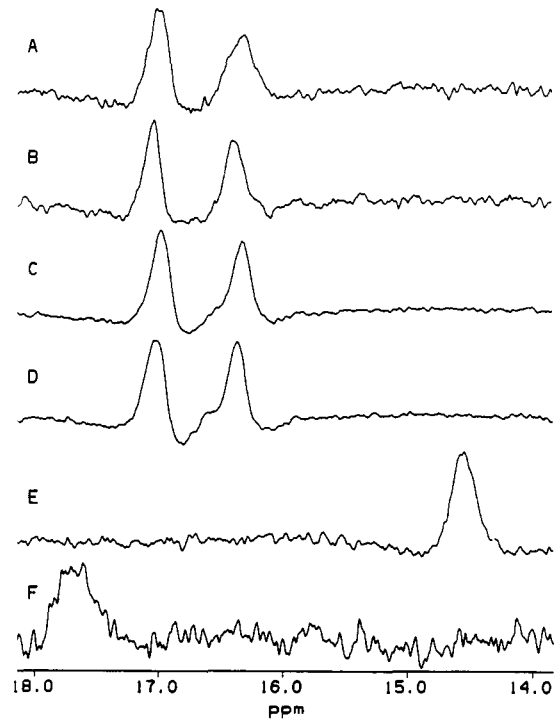

FIGURE 1: Low-field ^1H NMR of trypsin inhibited with (A) H-D-Val-Leu-boroArg, pH 9; (B) H-D-Val-Leu-boroArg, pH 5.1; (C) Ac-Gly-boroArg, pH 8.8; (D) Ac-Gly-boroArg, pH 5.7; and uninhibited resting enzyme (E), pH 9.1; (F) pH 5.6. The enzyme concentration was 2 mM, and each spectrum represents about 200 scans. The spectra were recorded at 278 K.

Table I: Low-Field ^1H NMR Chemical Shifts of Boronic Acid and Peptide Boronic Acid Complexes of Trypsin and Trypsinogen

complex	$N^{\delta 1}$-H	$N^{\epsilon 2}$-H	pH	adduct
trypsin +	14.5	*a*	>8.5	
	17.6	*a*	<6.5	
boric acid	15.8	*b*	~6.5–9.5	His
methane boronic acid	15.4	*b*	~6.5–9.5	His
butane boronic acid	15.8	*b*	~6.5–9.5	His
triethanolamine borate	15.7	*b*	~6.5–9.5	His
H-D-Val-Leu-boroArg	16.3	17	~3.5–10	Ser
Ac-Gly-boroArg	16.3	17	~3.5–10	Ser
trypsinogen +	14.5	*a*	>8.5	
	17.1	*a*	<6.5	
boric acid	15.4	*b*	~6.5–9.5	His
methane boronic acid	15.1	*b*	~6.5–9.5	His
butane boronic acid	15.4	*b*	~6.5–9.5	His
triethanolamine borate	15.5	*b*	~6.5–9.5	His
H-D-Val-Leu-boroArg	16.2	16.7	8.2	Ser
	16.1, 15.5	16.7	7.6	Ser, His
	16.1, 15.7	16.6	5.4	Ser,[c] His[c]
Ac-Gly-boroArg	16.1, 15.5	16.7	8.8	Ser, His
	16.1, 15.6	16.7	7.3	Ser,[c] His
	15.6	*b*	5.0	His
trypsinogen + Ile-Val +				
H-D-Val-Leu-boroArg	16.2	16.7	~4.5–9.5	Ser
Ac-Gly-boroArg	16.1, 16.3	16.6	~4.5–9.5	Ser
Ac-Gly-boroArg, 37 °C	16.2	16.8	8.7	Ser

[a] Does not apply because $N^{\epsilon 2}$-H is not observable under these conditions. [b] Does not apply because in histidine adducts $N^{\epsilon 2}$ does not have an attached proton. [c] Very weak signals for this complex.

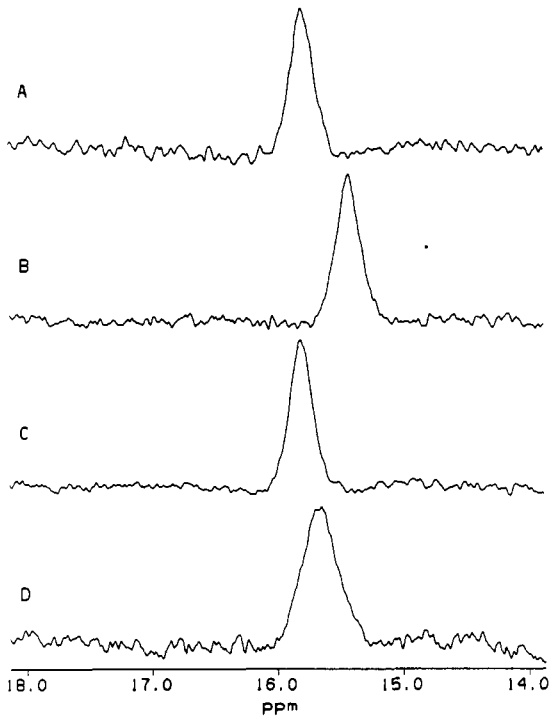

FIGURE 2: Low-field ^1H NMR of trypsin inhibited with (A) boric acid, pH 8.2; (B) methane boronic acid, pH 8.7; (C) butane boronic acid, pH 8.4; and (D) triethanolamine borate, pH 7.5. The enzyme concentration was 2 mM, and each spectrum represents about 500 scans. The spectra were recorded at 278 K.

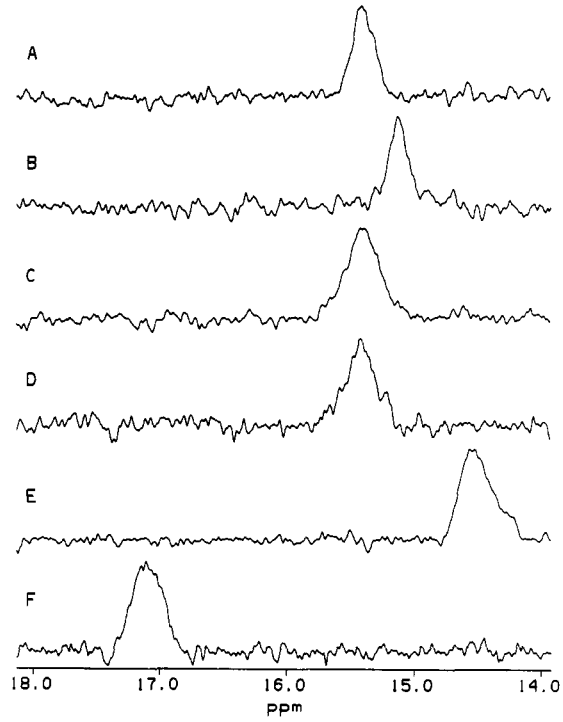

FIGURE 3: Low-field ^1H NMR of trypsinogen inhibited with (A) boric acid, pH 8; (B) methane boronic acid, pH 8.6; (C) butane boronic acid, pH 8.1; (D) triethanolamine borate, pH 8.3; and uninhibited resting enzyme (E), pH 9.1; (F) pH 4.4. The enzyme concentration was 2 mM, and each spectrum represents about 1000 scans. The spectra were recorded at 278 K.

protease, previously referred to as type 1 and shown to involve transition-state-like tetrahedral serine adducts. The similarities include (i) the presence of two low-field signals rather than one, (ii) the pH independence of both signals, (iii) the chemical shift separation of the signals, 0.7 ppm for trypsin versus about 0.5 ppm for the α-lytic protease complexes, and (iv) the chemical shift position of both signals located nearer to the low than the high pH position of the $N^{\delta 1}$-H protons in the respective resting enzymes. We therefore conclude that both H-D-Val-Leu-boroArg and Ac-Gly-boroArg form transition-state-like tetrahedral serine adducts with trypsin as depicted in structure **1** (Bachovchin et al., 1988). We tentatively assign the signals at 17.0 and 16.3 ppm to the protons on $N^{\epsilon 2}$ and on $N^{\delta 1}$, respectively, of His57 by analogy to α-lytic protease.

Complexes of trypsin with boric acid, methane boronic acid, butane boronic acid, and triethanolamine borate exhibit quite different low-field ^1H spectra from those of the peptide boronic acid complexes as illustrated in Figure 2. Each of these complexes shows a single, pH-independent resonance with a chemical shift ($\sim$15.6 ppm) about midrange between the low and high pH position of the signal from the uninhibited enzyme. These characteristics coincide with those exhibited by complexes of α-lytic protease with nonspecific boronic acid inhibitors shown to be histidine adducts. We therefore conclude that boric acid, methane boronic acid, butane boronic acid, and triethanolamine borate all form complexes with trypsin involving a tetrahedral histidine–boron adduct as illustrated in structure **2**. The histidine adduct complexes of trypsin are stable over a smaller pH range (pH 6.5–9.5, Table I) than the serine adduct complexes. Although these inhibitors bind much more weakly to trypsin than the peptide boroArg inhibitors, their complexes are nevertheless in slow exchange on the NMR time scale with resting enzyme. The spectra shown in Figure 2 are of samples to which sufficient inhibitor has been added to saturate the enzyme. Thus the chemical shifts of signals shown in Figure 2 represent that of each complex unaffected by exchange averaging with resting enzyme.

Figure 3 shows the low-field ^1H spectra of trypsinogen complexes with each of the above four nonspecific boronic acids. The spectra are quite similar to those of the corresponding trypsin complexes, i.e., a single, pH-independent signal with a midrange chemical shift. However, the chemical shifts of the low-field signals are not identical to those of the corresponding trypsin complexes. For each trypsinogen complex, the signal occurs 0.2–0.4 ppm upfield from its position in the corresponding trypsin complex (Table I). However, uninhibited trypsinogen also shows a difference of about the same magnitude and direction from uninhibited trypsin in the low pH position of the $N^{\delta 1}$-H proton. This signal occurs at about 17.6 ppm in trypsin (Figure 1F) and 17.1 ppm in trypsinogen (Figure 3F). The high pH positions of this signal do not differ significantly between trypsin and trypsinogen (Figures 1E and 3E). Thus, the systematic difference in the position of the low-field resonance between the trypsin and trypsinogen complexes likely reflects a difference in the environment of His57 between trypsin and trypsinogen which is preserved in each of the complexes. In any case, these results show that boric acid, methane boronic acid, butane boronic acid, and triethanolamine borate all form complexes with trypsinogen involving a histidine adduct, just as with trypsin.

The peptide boronic acid inhibitors, in contrast to the nonspecific boronic acid inhibitors, interact with trypsinogen very differently than with trypsin. Figure 4 summarizes the results obtained with Ac-Gly-boroArg and trypsinogen. The dominant feature in the spectra over the pH range from 5.0 to 8.0 (Figure 4B,C) is a single pH-independent resonance at 15.6 ppm characteristic of a histidine adduct. The weak signal

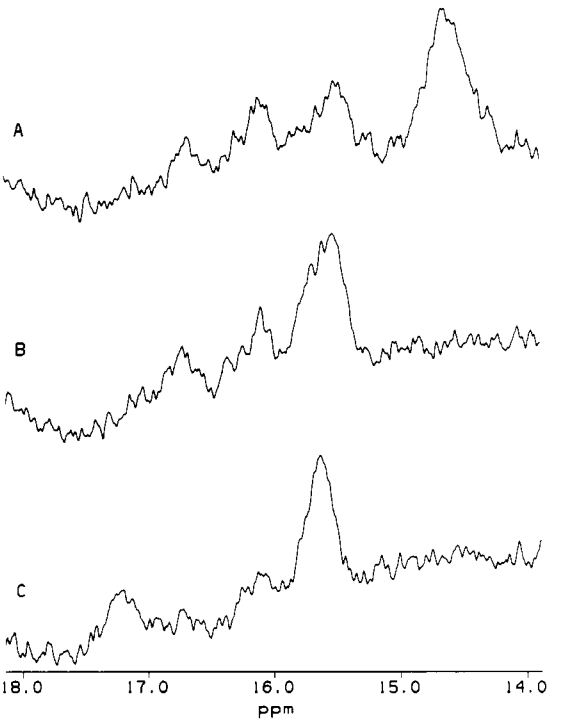

FIGURE 4: Low-field ^1H NMR of trypsinogen inhibited with Ac-Gly-boroArg. (A) pH 8.8; (B) pH 7.3; (C) pH 5. The enzyme concentration was 2 mM, and each spectrum represents about 1000 scans. The spectra were recorded at 278 K.

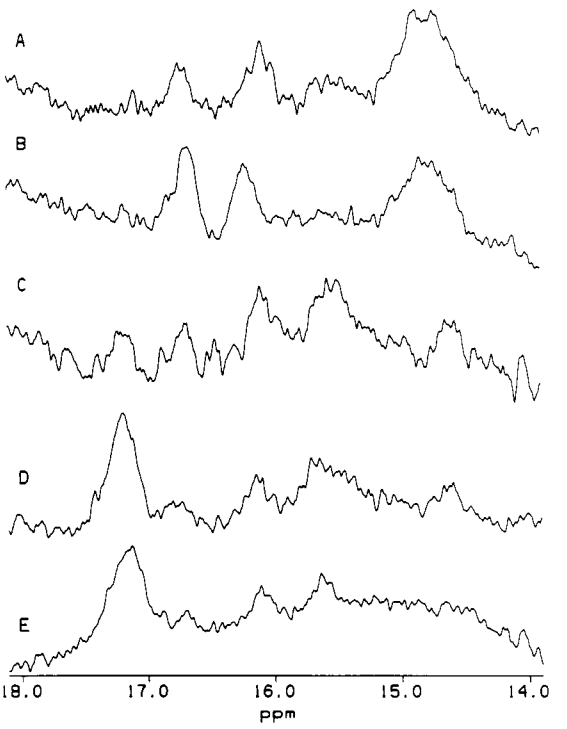

FIGURE 5: Low-field ^1H NMR of trypsinogen inhibited with H-D-Val-Leu-boroArg. (A) pH 8.3; (B) pH 8.2; (C) pH 7.6; (D) pH 6.4; (E) pH 5.4. The enzyme concentration was 2 mM, and each spectrum represents about 1000 scans. The spectra were recorded at 278 K.

at 17.2 ppm in the pH 5.0 spectrum (Figure 4C) is from resting enzyme in which His57 is fully protonated. At very high pH values, the 15.5 ppm signal, from the histidine adduct, becomes weaker, and a new signal at 14.6 ppm appears (Figure 4A) which is from the uninhibited enzyme in which His57 is fully neutral. Two other weak, pH-independent signals at 16.7 and 16.1 ppm can be observed in the pH 7.3 spectrum (Figure 4B), where they are small relative to the 15.6 ppm signal, and in the pH 8.8 spectrum (Figure 4A), where they have about the same intensity as the 15.5 ppm signal. These signals reflect the presence of a small amount of a serine adduct complex. The possibility that these two signals are due to the presence of a small amount of trypsin in the sample, which would form a serine adduct with the inhibitor, can be ruled out on the basis of their chemical shifts, which are about 0.3 ppm upfield from those of the authentic Ac-Gly-boroArg-trypsin complex (Figure 1C,D and Table I). Thus, these results show that Ac-Gly-boroArg, which binds tightly and stoichiometrically to trypsin as a transition-state-like tetrahedral serine adduct, binds to trypsinogen predominantly as a histidine adduct, but that a small amount of serine adduct complex is also formed.

Figure 5 summarizes the results obtained with trypsinogen and H-D-Val-Leu-boroArg. At high pH values, >8.0, the spectra (Figure 5A,B) show two weak, pH-independent signals at 16.7 and 16.1 ppm and a large signal at 14.8 ppm which represents resting enzyme. The presence of such a large amount of resting enzyme in the presence of 5-fold molar excess of the inhibitor demonstrates how much more weakly H-D-Val-Leu-boroArg binds to trypsinogen than to trypsin. It also demonstrates that in spite of the greatly weakened binding the complex is in slow exchange on the NMR time scale with resting enzyme. The chemical shifts of the two weak signals, about 0.3 ppm upfield from those of the H-D-Val-Leu-boroArg-trypsin adduct, coincide with those of the two weak signals observed in spectra of trypsinogen with Ac-Gly-boroArg. Thus, these results are consistent in indicating that serine adduct complexes of trypsinogen can be distinguished from serine adduct complexes of trypsin by the 0.3 ppm difference in chemical shifts. As the pH is lowered to 7.6, the signals indicative of a serine adduct begin to fade while a new signal at 15.5 ppm, indicative of a histidine adduct, appears (Figure 5C). As the pH is lowered further, a signal from resting enzyme at 17.1 ppm appears and dominates the spectrum, and only very small amounts of both histidine and serine adducts are observable (Figure 5D,E). Below pH 5 the inhibitor fully dissociates from the enzyme (data not shown). These results show that H-D-Val-Leu-boroArg binds much more weakly to trypsinogen than to trypsin and that, at physiological pH values, it forms both histidine and serine adducts with the histidine adduct slightly favored at pH values below 8.0 while at higher pH values the serine adduct is favored.

The addition of exogenous Ile-Val, the dipeptide formed at the amino terminus upon activation of trypsinogen to trypsin (Davie & Neurath, 1955; Desnuelle & Fabre, 1955), to the Ac-Gly-boroArg and to the H-D-Val-Leu-boroArg inhibited samples of trypsinogen has a dramatic effect on the low-field ^1H spectra as illustrated in Figure 6. The signals from the histidine adduct complex and from resting enzyme disappear entirely in the H-D-Val-Leu-boroArg complex while the signals at 16.7 and 16.2 ppm become much more intense (Figure 6D–F). A peculiarity is evident in the spectra of the Ac-Gly-boroArg complex (Figure 6A–C). Instead of two signals of equal intensity, the spectra show three signals, one at 16.7 ppm with the intensity of one proton and two with half-proton intensities at 16.3 and 16.1 ppm. This spectrum is maintained over the temperature range of 5–25 °C. However, if the temperature is raised to 37 °C, the two half-intensity signals merge into one signal of single-proton intensity and intermediate chemical shift (Table I). Apparently, the $N^{\delta 1}$-H proton, in this complex, exists in two slightly different states that interconvert slowly at room temperature, and this structural heterogeneity at $N^{\delta 1}$-H does not affect $N^{\epsilon 2}$-H. In any case, the results shown in Figure 6 demonstrate that the

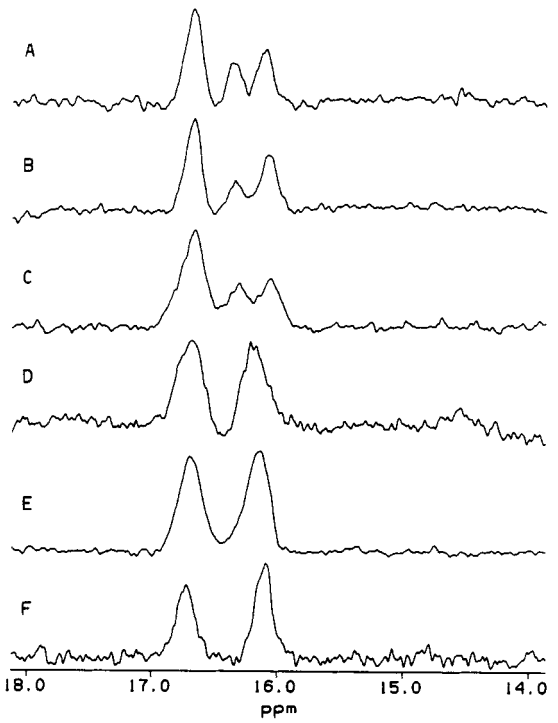

FIGURE 6: Low-field ^{1}H NMR of the complex Ile-Val–trypsinogen inhibited with Ac-Gly-boroArg, at (A) pH 8.8, (B) pH 7.4, and (C) pH 5.8, and inhibited with H-D-Val-Leu-boroArg at (D) pH 9, (E) pH 7.1, and (F) pH 4.8. The enzyme concentration was 2 mM, and each spectrum represents about 800 scans. The spectra were recorded at 278 K.

addition of Ile-Val transforms trypsinogen complexes with Ac-Gly-boroArg and H-D-Val-Leu-boroArg from predominantly histidine adducts into serine adducts. They also demonstrate, by the absence of signals from resting enzyme, that the inhibitors become more tightly bound in the presence of the Ile-Val dipeptide and that the complexes become more resistant to pH changes. The addition of Ile-Val does not, however, make the trypsinogen complexes indistinguishable from the corresponding trypsin complexes as the chemical shifts of the low-field protons of trypsinogen serine adducts stabilized by Ile-Val retain the ~0.3 ppm more upfield position from those of the corresponding trypsin complexes (Table I).

DISCUSSION

Two expectations formed in the previous study of α-lytic protease (Bachovchin et al., 1988) have been borne out in the present work. The first was that other serine proteases would also form histidine and serine adduct complexes with boronic acid inhibitors and that low-field ^{1}H NMR spectroscopy could be used to identify the type of complex formed in each case. It should be noted that the low-field ^{1}H spectra, by themselves, would be difficult or impossible to interpret. It was the ^{15}N NMR results that proved the existence of histidine and serine adducts and provided the structural details about these complexes which then showed how to interpret the low-field ^{1}H NMR spectra. The present study of trypsin demonstrates the remarkable clarity with which low-field ^{1}H NMR reveals and distinguishes between histidine and serine adducts and thus confirms its diagnostic value.

The second expectation was that the overall findings with trypsin would parallel those with α-lytic protease and show that only peptide boronic acid inhibitors able to make favorable contacts with the specificity subsites would form transition-state-like tetrahedral adducts with Ser195; the others would form histidine adducts. This work does indeed confirm this expection, showing that Ac-Gly-boroArg and H-D-Val-Leu-boroArg form serine adducts whereas boric acid, methane boronic acid, butane boronic acid, and triethanolamine borate form histidine adducts. The fact that trypsin has a very different primary specificity than α-lytic protease, cleaving after Arg and Lys compared to Ala and Val for α-lytic protease, makes trypsin a good test of the generality of the phenomenon. The clear confirmation obtained here with trypsin therefore supports the idea that the phenomenon is likely to be a general one, at least within the trypsin family of serine proteases. Nevertheless, further testing of the generality, by the examination of other serine proteases, is necessary and desirable. Such work with chymotrypsin, elastase, and subtilisin is in progress. Also highly desirable is a systematic investigation into the minimum structure required for inhibitors to permit formation of serine adducts, and work on this objective is also in progress.

In regard to the generality of this phenomenon, a systematic low-field ^{1}H NMR study of chymotrypsin and chymotrypsinogen inhibited with boric acid, benzene boronic acid, and phenylethane boronic acid has been previously reported (Robillard & Shulman, 1974b). The primary specificity of chymotrypsin is for Phe residues, so it is conceivable that benzene boronic acid and phenylethane boronic acid, although not peptide boronic acids, may be able to make sufficiently favorable contacts with the P_1 specificity subsite to form a serine adduct while boric acid should form a histidine adduct. However, the results showed only one pH-independent resonance with a midpoint chemical shift for all three complexes. Although originally interpreted in terms of serine adducts, we believe these spectra to be more indicative of histidine adducts although not conclusively because the chemical shifts of the single resonance in the benzene boronic acid and phenylethane boronic acid complexes are a little more downfield than those of histidine adducts of α-lytic protease and trypsin. Therefore the low-field ^{1}H NMR of chymotrypsin–boronic acid inhibitors needs to be reexamined.

What is the significance of the observation that serine adducts are only obtained with boronic acid inhibitors that are able to make favorable contacts with the enzyme's specificity subsites? As previously discussed in detail (Bachovchin et al., 1988), we proposed that occupancy of the specificity subsites induces the enzyme to assume a structure around Ser195 and the oxyanion hole that is more complementary to the transition state. We proposed that this effect is continuous, i.e., occupying more than one specificity subsite would have a larger effect on the transition-state binding site than occupying only one site, as would a more optimal, compared to a less optimal, filling of one subsite. If this hypothesis is correct, the conventional model of the resting enzyme as an essentially defined structure with a fully formed transition-state binding site and fully formed specificity subsites is incorrect. Instead, neither is fully formed, and the specificity subsites and transition state binding site bind their ligands synergistically, i.e., occupancy of the specificity subsites improves the ability of the transition-state site to bind a tetrahedral transition-state structure and vice versa, much like a positive allosteric effect in an allosteric protein.

This model nicely explains the otherwise very puzzling observation that small boronic acid inhibitors such as boric acid and methane boronic acid inhibit poorly and bind as histidine adducts, yet appropriate dipeptide and tripeptide

boronic acids, only marginally larger molecules, are among the most potent inhibitors of trypsin and α-lytic protease known, having K_i values in the subnanomolar range, and bind as serine adducts (Kettner et al., 1988). If the resting enzyme existed as a rigid molecule with a fully formed structure complementary to the transition state and fully formed specificity subsites, binding interactions in these sites should be more or less additive. If this model were correct, it is difficult to explain why the small boronic acids, with little or no structure to interfere with correct binding to the transition-state binding site, should bind so weakly and, moreover, bind as histidine rather than as serine adducts. Clearly, the binding interaction cannot be additive but instead must be synergistic. The model described here provides a mechanism to explain this apparent synergism. Thus far, structural evidence to support this model is lacking as X-ray crystallographic studies of α-lytic protease complexed to Boc-Ala-Pro-boroVal have been interpreted as indicating no significant structural changes in the enzyme upon binding the inhibitor (Bone et al., 1987). It should be noted, however, that this work did show small changes in the structure of the P_1 specificity subsite and in the structure of the oxyanion hole.

The present results with trypsinogen add strong support to the above model. Trypsin and trypsinogen are about 85% structurally homologous, the main structural differences residing in flexible portions of the proenzyme which become rigid and ordered upon conversion to trypsin (Huber & Bode, 1978). These regions include the specificity pocket and the oxyanion hole. Thus, in trypsinogen the catalytic triad is fully formed, but the specificity subsites and the oxyanion hole are not, a situation akin to what we are proposing for trypsin. There is, however, a difference. The trypsinogen to trypsin transition may be viewed as a continuum with the zymogen and the "active" enzyme representing opposite ends of the continuum. The trypsinogen structure is thus as far removed as possible along this continuum from the "active" structure and stabilized there by covalent bonds which are not present in any of the other structures along the continuum. Resting enzyme in this picture may be an intermediate which perhaps lies closer to the "active" end than to the zymogen end of the continuum but nevertheless does not have a structure fully complementary to the transition state. Ac-Gly-boroArg and H-D-Val-Leu-boroArg would then easily be able to induce the structural transition in resting trypsin to the "active" structure but not as able to induce this transition with trypsinogen. The net result is that these inhibitors form only serine adducts with trypsin but significant amounts of histidine adducts with trypsinogen. The small boronic acids are equally unable to induce a structural change in either trypsin or trypsinogen. The net result is that they only form histidine adducts with both. However, both Ac-Gly-boroArg and H-D-Val-Leu-boroArg form small amounts of serine adduct with trypsinogen. This indicates that both inhibitors are able, to some extent, to induce a structural transition all the way from trypsinogen to the "active" structure. Moreover, the tripeptide H-D-Val-Leu-boroArg is able to do this better than is the dipeptide boronic acid Ac-Gly-boroArg. This demonstrates that specificity subsite interactions are able to effect structural transition in the enzyme, that they do this in the correct direction, i.e., toward the "active" enzyme, and that tripeptide boronic acids are able to do this better than dipeptide boronic acids.

The induction of trypsinogen to a trypsin structure by trypsin inhibitors has been noted previously. BPTI and pGB have been shown to induce trypsinogen to adopt a trypsin-like structure (Huber et al., 1974; Kerr et al., 1975). A trypsinogen to trypsin-like transition is also induced by dipeptides sequentially related to the N terminus of trypsin in what is an allosteric-like effect as it does not involve binding to the active site (Bode & Huber, 1976). Trypsinogen is converted into trypsin by proteolytic removal of the amino-terminal hexapeptide (Davie & Neurath, 1955; Desnuelle & Fabre, 1955), generating Ile16–Val17 at the N terminus. The amino group of the newly formed N-terminal Ile16 then forms a salt bridge with Asp194 while the Ile side chain binds to a pocket (Huber & Bode, 1978). These interactions induce the structural transitions that result in the formation of the specificity subsites and the oxyanion hole or transition-state binding site. Exogenous Ile-Val dipeptide is apparently able to substitute for the N-terminal dipeptide in forming these interactions, thereby inducing trypsinogen to become more structurally similar to trypsin. Addition of Ile-Val to trypsinogen increases its k_{cat}/K_m value for the hydrolysis of trypsin specific substrates by 100–1000-fold (Antonini et al., 1984; Menegatti et al., 1985). Ile-Val also increases trypsinogen's affinity for BPTI, PSTI, and pGB (Bode, 1979; Antonini et al., 1983; Ascenzi et al., 1985, 1987; Coletta et al., 1990). Moreover, Bode and Huber (1976) reported that additional rigidification of pGB–trypsinogen and trypsinogen–BPTI with formation of the specificity pockets occurs upon addition of exogenous Ile-Val. Perkins and Wüthrich (1980) reported that the high-field ^1H NMR spectrum of PTI–trypsin was identical to that of PTI–trypsinogen when Ile-Val was added. These results demonstrate that the trypsin–trypsinogen system responds to binding interactions with structural changes and that more than just two structures represented by trypsin and trypsinogen are possible. Here, we have demonstrated that the addition of exogenous Ile-Val dramatically changes the way Ac-Gly-boroArg and H-D-Val-Leu-boroArg interact with trypsinogen, not only improving their affinity for trypsinogen but actually inducing the complexes to switch from histidine to serine adducts. Although we have not shown it, the reverse must also be true, i.e., binding of the peptide boronic acid to trypsinogen must improve its affinity for Ile-Val. The available evidence taken together strongly argues that trypsin is a flexible molecule able to exist in multiple conformations and that binding interactions in the specificity subsites, transition-state binding site, and Ile-Val binding site are all positively synergistic, with the synergy mediated through enzyme conformational changes.

The present work demonstrates that the phenomenon first observed with α-lytic protease, that occupancy of the specificity subsites is a necessary prerequisite for a boronic acid inhibitor to form a transition-state-like tetrahedral adduct with Ser195, is not unique to α-lytic protease. Trypsin exhibits the same phenomenon. This indicates that the phenomenon is likely to be general, at least within the trypsin family of serine proteases. On the basis of these results, we have proposed that native resting trypsin or α-lytic protease does not have a rigid, fully formed binding site complementary to the transition state. Formation of a site optimally complementary to the transition state requires occupancy of the specificity subsites. This mechanism is very similar to that proposed by Jencks (1969) on the basis of peptide binding studies and termed induced destabilization. Why serine proteases should have adopted such a mechanism is not clear at present because it does not seem to offer any advantage in terms of catalytic power, although it should offer advantages in terms of specificity.

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