Kinetic Properties of the Binding of α-Lytic Protease to Peptide Boronic Acids

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Received October 27, 1987; Revised Manuscript Received July 7, 1988

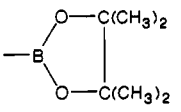
ABSTRACT: The kinetic parameters for peptide boronic acids in their interaction with α-lytic protease were determined and found to be similar to those of other serine proteases [Kettner, C., & Shenvi, A. B. (1984) *J. Biol. Chem. 259*, 15106-15114]. α-Lytic protease hydrolyzes substrates with either alanine or valine in the P_1 site and has a preference for substrate with a P_1 alanine. The most effective inhibitors are tri- and tetrapeptide analogues that have a -boroVal-OH residue in the P_1 site. At pH 7.5, MeOSuc-Ala-Ala-Pro-boroVal-OH has a K_i of 6.4 nM and Boc-Ala-Pro-boroVal-OH has a K_i of 0.35 nM. Ac-boroVal-OH and Ac-Pro-boroVal-OH are 220000- and 500-fold less effective, respectively, than the tetrapeptide analogue. The kinetic properties of the tri- and tetrapeptide analogues are consistent with the mechanism for slow-binding inhibition, $E + I \rightleftarrows EI \rightleftarrows EI^*$, while the less effective inhibitors are simple competitive inhibitors. MeO-Suc-Ala-Ala-Pro-boroAla-OH is a simple competitive inhibitor with a K_i of 67 nM at pH 7.5. Other peptide boronic acids, which are analogues of nonsubstrates, are less effective than substrate analogues but still are effective competitive inhibitors. For example, MeOSuc-Ala-Ala-Pro-boroPhe-OH has a K_i of 0.54 μM although substrates with a phenylalanine in the P_1 position are not hydrolyzed. Binding for boronic acid analogues of both substrate and nonsubstrate analogues is pH dependent with higher affinity near pH 7.5. Similar binding properties have been observed for pancreatic elastase. Both enzymes have almost identical requirements for an extended peptide inhibitor sequence in order to exhibit highly effective binding and slow-binding characteristics. Both enzymes have a greater than expected affinity for the nonsubstrate analogue terminating in boroPhe-OH.

Serine proteases are a large group of homologous enzymes that share a common reaction mechanism but have many diverse biological roles. Selective inhibition of a number of serine proteases could potentially serve as the basis of therapeutic agents. Recently, we have shown that peptide boronic acids are effective inhibitors of the serine proteases, chymotrypsin, and both pancreatic elastase and leukocyte elastase (Kettner & Shenvi, 1984). Inhibition of the latter enzyme is significant since it has been strongly implicated in the pathogenesis of emphysema (Hance & Crystal, 1975). One of the more effective boronic acid inhibitors of leukocyte elastase is effective in vivo in preventing porcine pancreatic elastase induced emphysema in hamsters (Soskel et al., 1986).

Our previous study demonstrated the utility of peptide boronic acids in the selective inhibition of serine proteases, and additional examples have been provided more recently by Kinder and Katzenellenbogen (1985). The foundation for this work has been provided by earlier mechanistic studies. Antonov et al. (1970) and Philipp and Bender (1971) observed that alkyl and aromatic boronic acid bind chymotrypsin and concluded that the boronic acids are forming an adduct with the active site histidine. Koeler and Lienhard (1971) in studies of the inhibition of chymotrypsin by aromatic boronic acids made observations consistent with the boronic acids acting as "transition-state analogues" by forming adducts with the active site serine. Matthews et al. (1975) concluded that aromatic boronic acids exist as a tetrehedral adduct with the active site serine in the crystal structure of subtilisin. A major advance in this area was the development of the chemistry to allow the preparation of the *N*-acetyl boronic acid analogue of phenylalanine and the demonstration that it was an effective inhibitor of chymotrypsin (Matteson et al., 1981).

In our previous studies with peptide boronic acids, we have shown that the kinetic properties of peptide boronic acid binding to serine proteases are unique in a number of ways and have left questions unanswered. Peptide boronic acids are reversible, competitive inhibitors of the serine proteases we have examined. MeOSuc-Ala-Ala-Pro-boroVal-OH[1] inhibits pancreatic elastase and leukocyte elastase with K_i's of 0.25-0.57 nM, exceeding most other synthetic reversible inhibitors in effectiveness. In addition, the peptide boronic acids, which are analogues of good substrates, are not simple competitive inhibitors but rather exhibit kinetic properties consistent with the mechanism for slow-binding inhibition (Williams & Morrison, 1979), $E + I \rightleftarrows EI \rightleftarrows EI^*$, where EI* is more stable than the EI complex. The molecular mechanisms for this slow-binding inhibition is unknown. In exam-

[1] Abbreviations: FAB-MS, fast-atom-bombardment mass spectrometry; NMR, nuclear magnetic resonance; pNA, *p*-nitroaniline; AMC, 7-amino-4-methylcoumarin; Ac, acetyl; Suc, succinyl; MeOSuc, methoxysuccinyl; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane. The prefix "boro" of -boroVal- indicates that the carbonyl of the amino acid residue, in this case a valyl residue, is replaced by $-B(OH)_2$; see Scheme II. -boroVal-pinacol indicates that the boronic acid is present as a pinacol ester:



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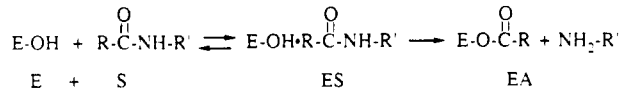
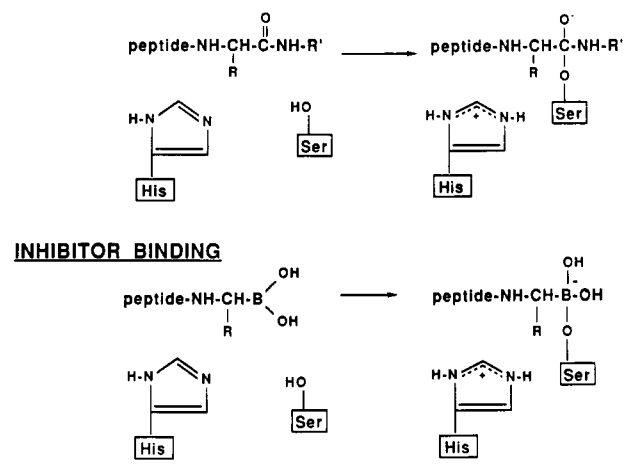
Scheme I



Scheme II



ining the binding of peptide boronic acids that are nonsubstrate analogues, we have found that they have a much greater affinity for the protease than would have been predicted. For example, Suc-Ala-Ala-Pro-Phe-pNA, a substrate for chymotrypsin-like proteases (Zimmerman & Ashe, 1977; Nakajima et al., 1979), is not hydrolyzed by the elastolytic enzymes although its boronic acid analogue has a K_i of 0.27 μM for pancreatic elastase.

We have continued the study of the binding of peptide boronic acids to serine proteases by studying their binding to α-lytic protease, an enzyme isolated from *Lysobacter enzymogenes* (Whitaker, 1970), which shares sequence and tertiary structure homology with elastase. It is similar to pancreatic elastase in its substrate specificity (Bauer et al., 1981). In addition, its crystal structure has been determined and refined to 1.7-Å resolution (Delaere et al., 1979; Fujinaga et al., 1985), demonstrating the presence of the Asp, His, and Ser catalytic residues common to all serine proteases. Because α-lytic protease contains only one histidine, it has been a favorite for NMR studies (Hunkapiller et al., 1973; Bachovchin & Roberts, 1978; Westler, 1980; Bachovchin, 1986).

In normal substrate hydrolysis (Bender & Kezdy, 1965), the protease binds substrate to form an ES complex, which is followed by nucleophilic addition of the active site serine to the substrate amide bond to yield an acyl enzyme, EA (Scheme I). This reaction is accompanied by the transfer of a proton to the active site histidine and then the transfer of this proton to the departing NH– group of the hydrolyzed bond. The transition from ES to EA occurs through a tetrahedral intermediate, which is expected to be mimicked by a tetrahedral complex of the boronic acid substrate analogues and the active site serine (Scheme II). Information obtained on the environment and properties of the active site histidine by ^{15}N NMR of α-lytic protease should yield direct evidence for the molecular mechanism of peptide boronic acid binding. Therefore, we have undertaken these studies. This paper describes the kinetic properties of binding of α-lytic protease to peptide boronic acids, and the following paper in this issue (Bachovchin et al., 1988) describes the effects of inhibitor binding on the active site histidine as determined by ^{15}N NMR. These studies have been conducted in combination with high-resolution X-ray crystal studies (Bone et al., 1987); thus three techniques that readily complement each other have been used in addressing basic questions on the mechanism of inhibitor binding.

Experimental Procedures

MeOSuc-Ala-Ala-Pro-(D,L)boroVal-pinacol, MeOSuc-Ala-Ala-Pro-(D,L)boroVal-OH, MeOSuc-Ala-Ala-Pro-(L)-boroAla-OH, and MeOSuc-Ala-Ala-Pro-(L)boroPhe-OH have been described previously (Kettner & Shenvi, 1984). Other peptide boronic acids were synthesized according to similar procedures and techniques.

Ac-(D,L)boroVal-pinacol was prepared by acylation of H-boroVal-pinacol·TFA with acetic anhydride. Anal. Calcd for $C_{12}H_{24}NO_3B$: C, 59.76; H, 10.05; N, 5.81; B, 4.48. Found: C, 59.57; H, 10.21; N, 5.64; B, 4.51.

Ac-Pro-(D,L)boroVal-pinacol was prepared by coupling Ac-Pro-OH to H-boroVal-pinacol according to the mixed anhydride procedure. After purification by silica gel chromatography using chloroform as a solvent, the product was obtained as a glass. NMR and FAB-MS were consistent with the desired structure. Anal. Calcd for $C_{17}H_{31}N_2O_4B·H_2O$: C, 57.30; H, 9.35; N, 7.86; B, 3.03. Found: C, 56.93; H, 9.57; N, 7.50; B, 2.85.

Boc-Ala-Pro-(D,L)boroVal-pinacol was obtained by coupling Boc-Ala-Pro-OH and H-boroVal-pinacol according to the mixed anhydride procedure and by purification by silica gel chromatography using chloroform as a solvent. Individual isomers were separated by crystallization. Boc-Ala-Pro-(D)-boroVal-pinacol crystallized from hexane (mp 131–133.5 °C) in a yield of approximately 50% from the material purified by chromatography. It was identified by X-ray structure analysis and by its lower reactivity in the inactivation of serine proteases. Anal. Calcd for $C_{23}H_{42}N_3O_6B$: C, 59.09; H, 9.07; N, 8.99; B, 2.31. Found: C, 59.37; H, 9.14; N, 8.92; B, 2.51.

Boc-Ala-Pro-(L)boroVal-pinacol was obtained by evaporation of the mother liquor from the above crystallization to yield a foam in analytically pure form on the basis of elemental composition and spectral properties. Note that the isomeric purity of this product is assumed to be close to 100% on the basis of the stoichiometric removal of the D isomer, which is sparsely soluble in hexane. Anal. Calcd for $C_{23}H_{42}N_3O_6B$: C, 59.09; H, 9.07; N, 8.99; B, 2.31. Found: C, 58.80; H, 8.99; N, 8.84; B, 2.54.

Boc-Ala-Pro-(D)boroVal-OH was prepared by transesterification of the corresponding pinacol ester with diethanolamine and by removing the diethanolamine ester by treatment with aqueous AG50-X8 (Bio-Rad) according to the procedure we have described previously, except the diethanolamine ester was purified by chromatography on Sephedex LH-20 in methanol. The desired product was obtained as an amorphous white solid. Anal. Calcd for $C_{17}H_{32}N_3O_6B$: C, 52.98; H, 8.39; N, 10.91; B, 2.80. Found: C, 53.76; H, 8.25; N, 10.16; B, 2.00. The corresponding pinanediol ester was prepared by incubation of the product with a 2-fold excess of pinanediol for 10 min in methanol and was analyzed by FAB-MS. Calcd for $M(C_{27}H_{46}N_3O_6B)$ + H: 520.36. Found: 520.47.

Pinacol protecting groups were removed from Ac-boro-Val-pinacol and from Ac-Pro-boroVal-pinacol with BCl_3 by a modification of the procedure of Kinder and Katzenellenbogen (1985), and the product was isolated by chromatography on a column of P-2 (Bio-Rad) in 10% acetic acid.

Inhibition constants for α-lytic protease were determined by using the substrates Ac-Ala-Pro-Ala-pNA (Hunkapiller et al., 1976) and Suc-Ala-Ala-Pro-Ala-7-amidino-4-methyl-

coumarin (Enzyme Systems Products). Assays were performed in 0.050 M sodium acetate, pH 5.5, 0.050 M Tris buffer, pH 7.5, and 0.050 M Tris buffer, pH 8.75; all buffers contained 0.10 M KCl. For the chromogenic assay using Ac-Ala-Pro-Ala-pNA, a series of cuvettes containing buffer, 4% ethanol, and 10.0, 4.00, 2.00, 1.30, and 1.00 mM substrate were incubated at 25 °C. The reactions were initiated by the addition of 10 μL of a stock solution of α-lytic protease in 0.10 M KCl to 1.00-mL solutions of substrate. The concentrations of protease in the assays were 0.10 μg/mL for reactions at pH 7.5 and 8.75 and 1.0 μg/mL for the reactions at pH 5.5. Activity was monitored by measuring the increase in absorbance at 405 nm on a Cary 219 spectrophotometer. Reactions were initiated simultaneously, and activity was measured for 30 min. Linearity in changes in absorbance with time was used as a criteria for the lack of slow-binding inhibition. For inhibitors that exhibited slow-binding inhibition, assays were initiated individually starting with the highest substrate concentration and initial velocities were measured at 5-min intervals. Final velocities were measured simultaneously 30 min after initiating the first assay. In all cases, final changes in absorbance with time were linear over a period of 20–30 min, indicating that steady-state velocities were being measured.

Assays using Suc-Ala-Ala-Pro-Ala-AMC were performed by a procedure similar to the chromogenic assays except ethanol was omitted from the assay buffer and the concentrations of substrate were 2.5, 0.75, 0.43, and 0.30 mM. Reaction rates were monitored on a Perkin-Elmer 650-40 fluorometer by excitation at 380 nm and by measuring fluorescence at 460 nm. Initial velocities were measured individually, and final velocities were measured individually after 30 min. The final concentrations of α-lytic protease in the assays were 0.20, 0.020, and 0.020 μg/mL for pH 5.5, 7.5, and 8.75, respectively.

Kinetic constants for the hydrolysis of substrates, K_m and V_m, were determined from double-reciprocal plots of velocity vs substrate concentration by the method of Lineweaver and Burk. Values of V_m in terms of molar concentrations of product were calculated by using an extension coefficient of 8800 M^{-1} cm^{-1} (Erlanger et al., 1961) for the chromogenic substrate and by using a standard curve prepared with 7-amino-4-methylcoumarin for the fluorogenic substrate. The concentration of protease was determined from the dry weight of lyophilized protein and a molecular weight of 19 900 (Olson et al., 1970). See titration data in Figure 2. Values of k_{cat} were calculated from V_m and the concentration of protease.

Inhibition constants for the inhibitors were also measured by the method of Lineweaver and Burk. Double-reciprocal plots of velocity vs substrate concentration were fitted to the best straight line by the least-squares procedure. Slopes of plots of initial velocities and of steady-state velocities measured in the presence of inhibitor were compared with velocities of controls run in the absence of inhibitor. These values were used to calculate values of K_i(initial) and of K_i(final). Values of k', the apparent, first-order rate constant for the transition from initial velocity to steady-state velocity, were determined by the fit of data to the equation of Cha (1975) as previously described (Kettner & Shenvi, 1984).

RESULTS

Substrate Hydrolysis. The kinetic properties for the hydrolysis of Suc-Ala-Ala-Pro-Ala-AMC and Ac-Ala-Pro-Ala-pNA are shown in Table I for the pH range from 5.5 to 8.75. Kinetic constants are in agreement with those measured previously at pH 8.75 (Hunkapiller et al., 1976) for the tripeptide substrate. For both substrates, the apparent second-order constant, k_{cat}/K_m, indicates the effectiveness of the protease in substrate hydrolysis is approximately equal at pH 7.5 and 8.75, which is the result of similar values of K_m and k_{cat}. The effectiveness of the protease is reduced 10–15-fold at pH 5.5, which is due to a corresponding decrease in k_{cat} for tetrapeptide substrate. A significant error was obtained in measuring V_m at pH 5.5 with the tripeptide substrate since the substrate was not soluble at levels required to saturate the enzyme. We estimate that the reduced value of k_{cat}/K_m is due to at least a 2-fold reduction in k_{cat} and an increase in K_m. The kinetic constants for the hydrolysis of Ac-Ala-Pro-Val-pNA were measured for comparison with the corresponding Ala-pNA (Table I, footnote *b*).

Table I: Kinetic Constants for Hydrolysis of Substrates by α-Lytic Protease[a]

pH	5.5	7.5	8.75
Suc-Ala-Ala-Pro-Ala-AMC			
K_m (mM)	2.0 ± 0.6	1.7 ± 0.2	2.3 ± 0.5
k_{cat} (s^{-1})	0.099 ± 0.017	1.3 ± 0.1	2.0 ± 0.2
k_{cat}/K_m (M^{-1} s^{-1})	52.2 ± 7.7	754 ± 90	877 ± 150
n	7	5	5
Ac-Ala-Pro-Ala-pNA[b]			
K_m (mM)		18 ± 4	16 ± 2
k_{cat} (s^{-1})		15 ± 7	19 ± 1
k_{cat}/K_m (M^{-1} s^{-1})	97 ± 25	1050 ± 110	1160 ± 130
n	4	5	5

[a] Kinetic constants were determined by the method of Lineweaver and Burk as described under Experimental Procedures. Kinetic constants are reported with the standard deviation for the indicated number of measurements, n. [b] Kinetic constants were measured for the hydrolysis of Ac-Ala-Pro-Val-pNA at pH 7.5 by using the exact conditions described for the corresponding -Ala-pNA. The following constants were measured and are reported as the average of three measurements: K_m = 42 ± 17 mM, k_{cat} = 2.2 ± 1 s^{-1}, and k_{cat}/K_m = 51 ± 5 M^{-1} s^{-1}.

The fluorogenic assay is approximately five times more sensitive and was used in many of our initial measurements of binding constants for the peptide boronic acids. However, Suc-Ala-Ala-Pro-Ala-AMC exhibits substrate inhibition at higher concentrations, and we were unable to demonstrate competitive inhibition with confidence. Despite this difficulty, a reasonable correlation was obtained between the apparent K_i's determined from the slopes of Lineweaver–Burk plots with Suc-Ala-Ala-Pro-Ala-AMC and the K_i's measured with Ac-Ala-Pro-Ala-pNA for peptide boronic acids which are substrate analogues (Table II, inhibitors I–IV and VI).

Kinetic Properties. The kinetic properties of the more effective inhibitors correspond to the mechanism of slow-binding inhibition. As shown for MeOSuc-Ala-Ala-Pro-boroVal-OH in Figure 1, when α-lytic protease is added to a solution of inhibitor and substrate, a progressive increase in inhibition is observed until a steady-state velocity is obtained. When the reaction was initiated by adding substrate to a preincubated solution of protease and inhibitor, a progressive increase in reaction velocity, or decrease in inhibition, is observed until a steady state is reached. Assays were performed by using the conditions in Figure 1 (curves A–C) at different substrate concentrations varied over a 10-fold range. Results indicate that MeOSuc-Ala-Ala-Pro-boroVal-OH is acting as a competitive inhibitor of α-lytic protease. For reactions initiated by the addition of enzyme as in curve B, y intercepts ($1/V_m$) of double-reciprocal plots of initial velocities (measured immediately after initiating the reaction) and of steady-state velocities vs substrate concentrations were within experimental error of the intercept of the plot performed in the absence of inhibitor (curve A). K_i(initial) and K_i(final) were then cal-

Table II: K_i Values for Binding of Peptide Boronic Acids to α-Lytic Protease[a]

inhibitor		pH 5.5		pH 7.5		pH 8.75	
		K_i(initial)	K_i(final)	K_i(initial)	K_i(final)	K_i(initial)	K_i(final)
I	Ac-boroVal-OH		1.5×10^7 (5.0×10^4)		1.4×10^6 (2.9×10^5)		1.3×10^6 (2.2×10^5)
II	Ac-Pro-boroVal-OH		45000 (1600)		3300 (30)		5200 (78)
III	MeOSuc-Ala-Ala-Pro-boroVal-OH	590 (120)	72 (2.4)	68 (6.7)	6.4 (1.0)	97 (6.8)	11.0 (1.6)
IV	MeOSuc-Ala-Ala-Pro-(L)boroAla-OH		290 (16)		67.0 (1.0)		420 (2.0)
V	MeOSuc-Ala-Ala-Pro-(L)boroPhe-OH		2700 (140)		540 (63)		2700 (45)
VI	Boc-Ala-Pro-(L)boroVal-OH		11 (5.5)	4.3 (0.95)	0.35 (0.11)	22	1.0 (0.200)
VII	Boc-Ala-Pro-(D)boroVal-OH		6800 (1000)	1500	620[c] (100)		
VIII	Boc-Ala-Pro-(D)boroVal-pinacol	440000 (55000)	96000 (1000)	20000 (3100)	8600 (840)	60000 (11000)	8800 (280)
IX	phenylboronic acid		1.5×10^7 (3.9×10^6)		1.1×10^6 (2.3×10^5)		2.3×10^6 (4.6×10^5)

[a] K_i(initial) and K_i(final) values were determined by the methods described under Experimental Procedures and are reported as nanomolar concentrations. Standard deviations are shown in parentheses for at least duplicate measurements. The absence of data for K_i(initial) indicates that slow-binding inhibition was not observed, except for VI, in which the onset of inhibition at pH 5.5 was too rapid to determine K_i(initial) accurately. K_i's were determined with Ac-Ala-Pro-Ala-pNA as a substrate, except for VI, in which Suc-Ala-Ala-Pro-Ala-AMC was used. The concentrations of protease were 50, 5.0, and 5.0 nM for pH values 5.5, 7.5, and 8.75, respectively. The following concentrations of inhibitor were used to determine K_i values and are reported for each pH column as compound number (concentration): for pH 5.5, I (7.0 mM), II (20 μM), III (0.25 μM), IV (0.40 μM), V (5.0 and 10 μM), VI (50 nM), VII (10 μM), VIII (200 μM), and IX (10 mM); for pH 7.5, I (1.4 mM), II (5.0 μM), III (25 nM), IV (0.10 μM), V (0.50 μM), VI (5.0 nM), VII (1.0 μM), VIII (10 μM), and IX (2.0 mM); for pH 8.75, I (1.4 mM), II (10 μM), III (25 nM), IV (0.50 μM), V (5.0 μM), VI (5.0 nM), VIII (20 μM), and IX (2.0 mM). The following apparent K_i values for III were measured with the substrate Suc-Ala-Ala-Pro-Ala-AMC and reported as pH [K_i(initial), K_i(final)]: pH 5.5 (970 ± 150 nM, 58 ± 5 nM), pH 7.5 (38 ± 15 nM, 4.0 ± 0.6 nM), and pH 8.75 (59 ± 20 nM, 5.7 ± 0.6 nM). The concentrations of inhibitor were 150, 25, and 15 nM, and final concentrations of protease were 10, 1.0, and 1.0 nM for determinations at pH 5.5, 7.5, and 8.75, respectively. [b] The boroVal-OH residues were all in the D,L configuration unless specified, and no correction was made for the lower reactivity of the D isomer. Also, the boroVal-OH inhibitor (II, III, VI, and VIII) were in the form of their pinacol esters and were incubated in 0.10 M sodium phosphate buffer, pH 7.5, for at least 30 min prior to running the assay to hydrolyze the pinacol ester as we have described previously (Kettner & Shenvi, 1984). Note that VIII appears to be an exception and is bound as its pinacol ester (see Results). The remaining inhibitor (IV and V) were in the L configuration and were the free boronic acid. [c] The K_i(final) reported is an average of the constants measured by the usual procedure, initiating the reaction by adding enzyme to a solution of substrate and inhibitor (1.0 μM), and by initiating the reaction by diluting a solution enzyme and inhibitor (10 μM) 10-fold with substrate.

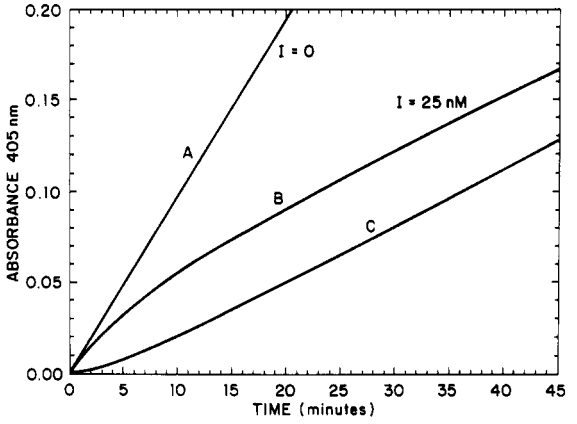


FIGURE 1: Effects of MeOSuc-Ala-Ala-Pro-boroVal-OH (I) on the hydrolysis of Ac-Ala-Pro-Ala-pNA (S) by α-lytic protease (E). Assays were run at pH 7.5 and 25 °C; the final concentrations of reactants were [E] = 5.0 nM, [S] = 4.0 mM, and [I] = 25.0 nM. Curve A is the increase in absorbance after adding E to S in the absence of I. Curve B is the same as curve A except I was incubated with S prior to adding E. For curve C, E and I were preincubated for 5 min and diluted 100-fold into a solution of S to initiate the reaction. For curve B, k' was 0.15 min^{-1}, and for curve C, k' was 0.20 min^{-1}.

culated for the respective initial velocity and final velocity measurements. Similar values of K_i(final) or the steady-state inhibition constants were obtained from enzyme-initiated assays (curve B) and from those in which the enzyme and inhibitor were preincubated (curve C). More details of the binding of MeOSuc-Ala-Ala-Pro-boroVal-OH are given in Table II, in which results are reported for multiple measurements.

It should also be noted that when Ac-Ala-Pro-Ala-pNA is used as a substrate for the reaction of MeOSuc-Ala-Ala-Pro-boroVal-OH with the protease, the insensitivity of the assay does not allow a low enough concentration of enzyme to provide a wide margin for maintaining pseudo-first-order reaction conditions. However, the apparent K_i values measured with the more sensitive fluorogenic substrate, in which a larger excess of inhibitor over enzyme was maintained, are within the range we have observed when comparing these two assays.

Stoichiometry of Peptide Boronic Acid Binding. The most effective peptide boronic acid, Boc-Ala-Pro-boroVal-OH, was used to confirm that a 1:1 complex is formed with the inhibitor and protease by using the titration procedure of Morrison (1969). The protease was allowed to react with substoichiometric levels of inhibitor and at concentrations greater than K_i. As shown in Figure 2, Boc-Ala-Pro-(L)boroVal-OH reacts stoichiometrically with the enzyme. The intercept on the x axis for 440 nM protease is 448 nM.

Inhibitor Binding. The inhibition constants for boronic acids with α-lytic protease at three different pH values are shown in Table II. Inhibition is described by values of K_i(initial) and K_i(final) for inhibitors that exhibited slow-binding inhibition and by K_i(final) for inhibitors that behaved as simple competitive inhibitors. In all cases inhibition appears to be competitive; however, it should be noted that at pH 5.5 double-reciprocal plots intercepted the y axis close to the origin, making it difficult to determine the nature of inhibition accurately, particularly for the more effective inhibitors. Similarly, it was not possible to determine the nature of inhibition of Boc-Ala-Pro-(L)boroVal-OH (VI) with certainty due to its high affinity for the protease and required use of the fluoro-

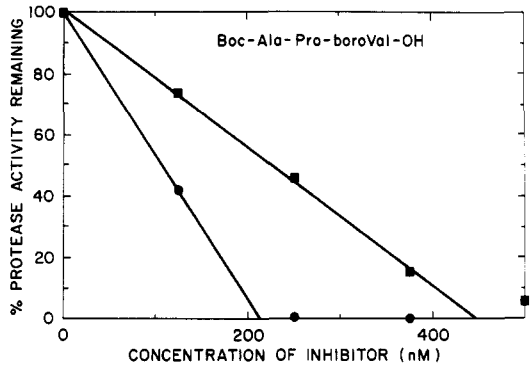


FIGURE 2: Titration of α-lytic protease. Protease activity of α-lytic protease (E) was titrated with Boc-Ala-Pro-(L)boroVal-OH by incubating the indicated concentrations of inhibitors with either 220 nM or 440 nM E (concentrations based on weight of lyophilized protein) in buffer (0.10 M sodium phosphate buffer, pH 7.5, containing 0.50 M NaCl at 25 °C) for 5 min; diluting 10-fold with buffer containing 1.10 mM Ac-Ala-Pro-Ala-pNA as a substrate; and measuring the initial activity, monitored by the increase in absorbance at 405 nm. Activity in the absence of inhibitor was 0.046 min^{-1} and 0.022 min^{-1} for the 440 and 220 nM protease samples, respectively. Points on the titration curve are shown as (●) for 220 nM enzyme and as (■) for 440 nM enzyme.

genic substrate which had complex saturation kinetics as previously discussed. It seems unlikely that VI is not a competitive inhibitor, especially since it binds stoichiometrically (Figure 2) with exceptionally low K_i.

Compounds II, III, and VI were tested after removing the pinacol esters in situ by incubation at pH 7.5 in phosphate buffer for 1 h. This procedure is effective in removing the pinacol group from III, yielding solutions of inhibitor several orders of magnitude more effective in initial binding and comparable to the free boronic acid in reactivity (Kettner & Shenvi, 1984). This was confirmed for inhibitors I and II in this study. The pinacol ester form of I, after incubation in phosphate buffer for 1 h, yielded inhibition constants that were almost identical with those measured with the free boronic acid at pH 5.5 and 8.75. Single measurements of the inhibition constants for the free boronic acid form of II (see Experimental Procedures) at all three pH values and the free boronic acid form of III at pH 7.5 were within experimental error of those reported in Table II. A clear exception to this observation is the inhibitor Boc-Ala-Pro-(D)boroVal-pinacol (VIII), which after preincubation in phosphate buffer results in only a slight increase (approximately 10%) in inhibitory activity, and no additional change is observed with further incubation (24 h). The free boronic acid, prepared by removing the pinacol ester through a series of chemical steps and characterizing the product (Experimental Procedures), is at least ten times more effective. VIII exhibits slow-binding inhibition similar to the results in Figure 1 for III. Comparable values of K_i were obtained when K_i's were measured according to the two methods illustrated in Figure 1 at pH 7.5. The possibility exists that a small degree of racemazation occurred during the removal of the pinacol group of VIII, which would yield the most effective inhibitor, VI. This is unlikely since measurements made after initiating reactions by adding substrate to preincubated solutions of enzyme and inhibitor as described in Figure 1 (curve C) would result in VI acting as a titrant of the enzyme at the level used for the determination of K_i. These results suggest that the pinacol ester of Boc-Ala-Pro-(D)-boroVal-pinacol is unusually stable.

Compounds I–III are used to assess the effect of increasing the peptide chain length of boroVal inhibitors on binding. As shown by comparing I and II, a 250–300-fold increase in affinity is obtained by binding of Pro in the P_2 site, and by comparing I and III, a 120 000–220 000-fold increase in affinity was obtained by binding MeOSuc-Ala-Ala-Pro- in the P_4–P_2 sites. These results were consistent over the 5.5–8.75 pH range. Inhibitors I and II behave as simple competitive inhibitors, while III is a slow-binding inhibitor exhibiting approximately a 10-fold difference in K_i(initial) and K_i(final) for the pH range used. Boc-Ala-Pro-(L)boroVal-OH is the most effective inhibitor for α-lytic protease binding, with an apparent K_i of 3.5×10^{-10} M, and exhibits slow-binding properties. The preference of the protease for VI indicates the presence of a hydrophobic binding pocket in the region of the P_4 binding site.

In the series III–VI, comparison of the binding properties of MeOSuc-Ala-Ala-Pro-boroVal-OH (III) with those of MeOSuc-Ala-Ala-Pro-boroAla-OH (IV) and MeOSuc-Ala-Ala-Pro-boroPhe-OH (V) assesses the influence of the P_1 side chain on inhibitor binding. The boroVal peptide III is the most effective inhibitor, binding ten times more tightly than the corresponding boroAla peptide (IV) and binding approximately one hundred times more tightly than the corresponding boroPhe peptide (V). In contrast to the slow-binding boroVal peptide, IV and V are simple competitive inhibitors. It should be noted that the boroPhe peptide is still an effective inhibitor, with a K_i of 5.4×10^{-7} M.

pH Profile. Values of k_{cat}/K_m for substrate hydrolysis increased 9–15-fold in going from pH 5.5 to 7.5 while they changed little between pH 7.5 and 8.75 (Table I). Comparison of the K_i valves for compounds I–III at the three pH values with values of k_{cat}/K_m shows a similar trend. In addition, a similar trend is observed for phenyl boronic VIII.

Compounds IV and V, the boroAla and boroPhe peptides, exhibited a unique pH profile in which about five fold greater affinity was observed at pH 7.5 than pH 5.5 or 8.75.

Discussion

The binding of α-lytic protease was studied with a number of boronic acids which we have divided into two groups, substrate analogues and nonsubstrate analogues, on the basis of reported specificity of α-lytic protease (Bauer et al., 1981) and by analogy drawn from pancreatic elastase (Thompson & Blout, 1973; Nakajma et al., 1979) and other serine proteases. The substrate analogues terminate in (L)boroVal or (L)boroAla residues, and the nonsubstrate analogues terminate in boroPhe or (D)boroVal and phenylboronic acid. [In the following paper (Bachovchin et al., 1988), most substrate analogues are classified as "type 1" inhibitors, and nonsubstrate analogues are classified as "type 2" inhibitors on the basis of NMR spectral properties of inhibitor–protease complexes.]

Binding Substrate Analogues. For the substrate analogues, we have observed that when the length of the peptide chain is increased in the series Ac-boroVal-OH, Ac-Pro-boroVal-OH, and MeOSuc-Ala-Ala-Pro-boroVal-OH, the compounds are competitive inhibitors of α-lytic protease and that inhibitor effectiveness increases with the length of the peptide chain by 5 orders of magnitude. Boc-Ala-Pro-(L)boroVal-OH is even more effective than the tetrapeptide analogue due to favorable interactions of the Boc- group in the P_4 binding site (Bone et al., 1987). This inhibitor binds α-lytic protease with a K_i of less than 1.0 nM, and at higher concentrations, it acts as a titrant of the enzyme. Slow-binding inhibition was observed only for the more effective inhibitors, Boc-Ala-Pro-(L)boro-Val-OH and MeOSuc-Ala-Ala-Pro-boroVal-OH.

Inhibition constants were measured at three pH values, chosen as typical points on the pH profile of serine protease in which differences between pH 5.5 and 7.5 reflect the pK_a

of the active site histidine, which has a pK_a of 7.0 (Bachovchin & Roberts, 1978). Measurement at pH 8.75 corresponds to the alkaline plateau obtained in the titration of this histidine by ^{15}N NMR. Comparisons of inhibitor binding at the three pH values for substrate analogues indicate that maximum binding was at pH 7.5 for all inhibitors. At pH 5.5, inhibitor binding is reduced 10-fold for all inhibitors except for Boc-Ala-Pro-(L)boroVal-OH, in which a 30-fold reduction is observed. No significant difference was observed in the binding of Ac-boroVal-OH at pH 7.5 and 8.75. For the remaining inhibitors in this series, small reductions in binding at pH 8.75 are observed with the increase in inhibitor effectiveness. For example, the ratio of K_i(final) values at pH 8.75 and 7.5 in the (L)boroValine series, Ac-, Ac-Pro-, MeOSuc-Ala-Ala-Pro-, and Boc-Ala-Pro- increases from 1 to 3.

Comparisons of Inhibitors with Substrates. Extensions of the length of the boroVal-containing peptide to the P_2, P_3, and P_4 binding sites result in increased binding affinity, analogous to the increased k_{cat}/K_m observed for the Ala-pNA reported by Bauer et al. (1981). The preference of α-lytic protease for the hydrophobic *tert*-butyloxycarbonyl (Boc) group in the region of the P_4 position has been observed with substrates for pancreatic elastase (Harper et al., 1984) and probably reflects structural similarities of these enzymes at the P_4 site. Further comparisons of substrate hydrolysis and inhibitor binding can be made in the pH profiles of substrate hydrolysis. The values of k_{cat}/K_m of the hydrolysis of Ac-Ala-Pro-Ala-pNA were 10-fold lower at pH 5.5 than pH 7.5 and did not change between pH 7.5 and 8.75. This corresponds well with inhibitor binding for the boroVal inhibitors, except the more effective compounds in this series had a small reduction in affinity at pH 8.75. The foregoing results are consistent with the correlations between inhibitor binding to serine proteases for peptide aldehydes and values of k_{cat}/K_m for substrate hydrolysis (Thompson & Bauer, 1979). This might be expected since peptide aldehydes are felt to behave as reaction-intermediate analogues, like the peptide boronic acids.

However, the analogy between substrate hydrolysis and inhibitor binding is not observed when one compares the binding of the substrate analogues terminating in boroVal and boroAla. α-Lytic protease hydrolyses substrates with an alanyl residue in the P_1 site more effectively than those with a valyl residue. For example, Bauer et al. have shown that Ac-Pro-Ala-Pro-Ala-NH_2 is hydrolyzed three times more effectively than the corresponding Val-NH_2, and we have observed a 20-fold preference for the hydrolysis of the Ala-pNA bond of Ac-Ala-Pro-Ala-pNA when compared with the corresponding Val-pNA. In contrast to substrate hydrolysis, the affinity of MeOSuc-Ala-Ala-Pro-boroAla-OH is approximately 10-fold less than that of the corresponding boroVal-OH peptide. Furthermore, the binding of MeOSuc-Ala-Ala-Pro-boroAla-OH is less dependent on pH than is substrate hydrolysis and binding of the boroVal inhibitors. These observations suggest that the P_1 binding site of α-lytic protease can readily accommodate a branched-chain amino acid residue such as the valyl side chain, but factors leading to stabilization of the boroVal–enzyme complex are not present in catalysis.

Binding of Nonsubstrate Analogues. The ability of nonsubstrate analogues to bind α-lytic protease is a property that cannot be explained without additional physical data. Phenylboronic acid binds α-lytic protease with an affinity and pH profile similar to those of the substrate analogue Ac-boroVal-OH, although one cannot draw any analogy between its structure and that of the substrate analogue except for the presence of the trigonal boronic acid. The K_i of MeOSuc-Ala-Ala-Pro-boroPhe-OH at pH 7.5 is 0.53 μM, only ten times less effective than the corresponding boroAla, but one hundred times greater than that of the corresponding boroVal inhibitor. The stereochemistry of the boroPhe residue is also important since MeOSuc-Ala-Ala-Pro-(D)boro-Phe-OH is approximately fifty times less effective (data not shown). Boc-Ala-Pro-(D)boroVal-OH also is an effective inhibitor of α-lytic protease and, in addition, exhibits slow-binding properties although less than for the substrate analogues.

Slow-Binding Properties. MeOSuc-Ala-Ala-Pro-boroVal-OH is a slow-binding inhibitor with kinetic properties similar to those obtained previously for peptide boronic acid binding to serine proteases and consistent with the mechanism for slow-binding inhibition (Kettner & Shenvi, 1984). Over the pH range of 5.5–8.75, a consistent 10-fold difference in K_i(initial) and K_i(final) was measured (Table II). Of the other proteases we have examined, a 20–100-fold difference was observed at pH 7.5. The ratio of K_i(initial)/K_i(final) is a measure of the slow-binding properties of the inhibitor. This ratio, equal to $k_3/k_4 + 1$ (see eq 2), is defined by the ratio of the slow steps of the mechanism, where k_3 is the rate constant for formation of EI* from EI and k_4 is the rate constant for its dissociation to EI (Schloss et al., 1980). These

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^*$$

$$K_i(\text{initial}) = k_2/k_1 \quad (1)$$

$$K_i(\text{initial})/K_i(\text{final}) = k_3/k_4 + 1 \quad (2)$$

similarities suggest that α-lytic protease and other serine proteases share a common molecular mechanism for slow-binding inhibition. Furthermore, the consistent ratio of K_i(initial)/K_i(final) for MeOSuc-Ala-Ala-Pro-boroVal-OH with changes in pH suggests that the molecular events leading to this process have no large pH dependence.

Overview. Crystal structure studies of α-lytic protease and pancreatic elastase have shown 55% topological homology and little difference in the positions of the catalytic residues, but a significant number of differences in the residues comprising the P_1 binding site have been observed (Delbaere et al., 1979; Fujinaga et al., 1985). Comparison of our data for peptide boronic acid binding for α-lytic protease with data for pancreatic elastase and for leukocyte elastase revealed similarities with both enzymes. α-Lytic protease has a lower affinity for the peptide boronic acids and has a lower catalytic efficiency. Most notable similarities are the requirement of a peptide chain extended to the P_3 binding site to exhibit slow-binding of properties (unpublished results) and higher than expected affinity of nonsubstrate analogues. These properties indicate that α-lytic protease will be a useful model system to study the effects of peptide boronic acid binding on the catalytic residue in the active sites of serine proteases.

The foregoing study has provided kinetic characterizations of the interactions of peptide boronic acids with α-lytic protease. These studies have been done in conjunction with X-ray crystallographic studies (Bone et al., 1987) and NMR studies (Bachovchin et al., 1988). Both have obtained results showing the formation of a tetrahedral adduct between the active site serine and the boronic acid moiety of substrate analogues. For nonsubstrate analogues, NMR studies have identified a novel mode of binding of the boronic acid with catalytic residues which appears to be consistent for all compounds falling into this group. Similar results have been obtained from X-ray crystallographic studies (Bone and Agard, unpublished results). Crystallographic studies presently in progress are expected to

provide an insight to the contribution of the peptide portion of nonsubstrate analogues in binding. Through the consolidation of results obtained from NMR and X-ray crystal studies, we anticipate the identification of molecular events occurring in inhibitor binding in even greater detail, such as those leading to slow-binding inhibition and their relationship to catalysis.

ACKNOWLEDGMENTS

We express our appreciation to Lawrence Mersinger for his excellent technical assistance and to Ashok Shenvi for useful discussions and for providing α-aminoboronic acids used as starting material for the synthesis in the initial phase of this work. Thanks are extended to David Anton and John Schloss for reviewing the manuscript and for their helpful suggestions.

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