Inhibition of the Serine Proteases Leukocyte Elastase, Pancreatic Elastase, Cathepsin G, and Chymotrypsin by Peptide Boronic Acids*

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Three α-aminoboronic acid-containing analogs of good peptide substrates for serine proteases were synthesized, MeO-Suc-Ala-Ala-Pro-boro-Phe-OH, MeO-Suc-Ala-Ala-Pro-boro-Ala-OH, and MeO-Suc-Ala-Ala-Pro-boro-Val-OH. They were effective inhibitors of chymotrypsin, cathepsin G, and both leukocyte and pancreatic elastase at nanomolar concentrations (0.10–20 nM). Except for cathepsin G, inhibition was not simply competitive, but showed kinetic properties corresponding to the mechanism for slow-binding inhibition, i.e. \( E + I \rightleftharpoons EI = EI^* \), where \( EI \) and \( EI^* \) are enzyme-inhibitor complexes and \( EI^* \) is more stable than \( EI \). This type of inhibition has not been observed previously for synthetic inhibitors or serine proteases and in this study it was observed only for peptide boronic acids which satisfy the primary specificity requirements of the protease.

Leukocyte elastase and cathepsin G are serine proteases associated with the granular fraction of polymorphonuclear leukocytes which hydrolyze multiple bonds of protein substrates. Particular emphasis has been placed on the study of leukocyte elastase due to its strong implication in diseases such as emphysema and septicemia. Its role in emphysema is the aberrant proteolysis of lung elastin (Mittman, 1972; Turino et al., 1974; Hance and Crystal, 1975) while in septicemia, elevated plasma levels of elastase associated with infection suggest that it is involved in nonspecific degradation of plasma proteins (Jochum et al., 1983). The role of cathepsin G in disease processes is less clear, but its ability to degrade proteoglycans and elastin suggest that it may also be involved in emphysema (Reilly and Travis, 1980). Cathepsin G also liberates the hypertensive peptide angiotensin II from angiotensin I (Reilly et al., 1982) and from angiotensinogen (Wintroub et al., 1984); however, the physiological significance of these reactions is unclear. Therefore, specific inhibitors of leukocyte elastase and cathepsin G may be useful therapeutically in the control of emphysema and septicemia as well as in determining the physiological significance of other reactions catalyzed by cathepsin G and elastase.

A number of synthetic inhibitors of leukocyte and pancreatic elastase and of cathepsin G and chymotrypsin are known. These inhibitors include peptide chloromethyl ketones (Powers et al., 1977), azapeptides (Powers and Carroll, 1975), trifluoroacetamide peptides (Renaud et al., 1983), aroylsulfonfyl fluorides (Yoshimura et al., 1982), and heterocyclic agents (Zimmerman et al., 1990; Teshima et al., 1982). \( K_i \) values for the more effective inhibitors obtained in these studies range from 0.10 to 0.010 μM (see Teshima et al., 1982, for comparisons).

The approach we have taken in a search for more selective and effective inhibitors of the leukocyte proteases pancreatic elastase and chymotrypsin has been to prepare peptide boronic acids. This group of compounds takes advantage of binding in the P1–P6 sites (using the nomenclature of Schecter and Berger, 1967) and of the binding of the boronic acid which can potentially act as a "transition state analog." Selection of the residues in the P1–P6 binding sites of the peptide boronic acids was based on the amino acid sequence of the better substrates of these enzymes from the extensive specificity studies of Zimmermann and Ashe (1977), Nakajima et al., (1979), and McRae et al. (1980). The proposed mechanism of binding of peptide boronic acids is shown in Fig. 1.

The serine proteases chymotrypsin and subtilisin have been shown to be inhibited by aromatic boronic acids (Koehler and Lienhard, 1971; Lindquist and Terry, 1974). The crystal structure of subtilisin complexed to either phenylethane boronic acid or phenyl boronic acid has been determined (Matthews et al., 1975). In the crystal structure, the trigonal boronic acid exists as a transition-state-like, tetrahedral adduct with the active site serine of the protease. One -OH of boron occupies the oxygen hole and the other -OH corresponds to the expected position of the departing NH group for normal hydrolysis.

Matteson et al. (1981) have further demonstrated the potential of boronic acids as serine protease inhibitors by the preparation of the boronic acid analog of N-acetyl-phenylal-
amine, (R)-acetamido-2-phenylethylboronic acid. They have shown that this compound is effective in the micromolar concentration range and is several orders of magnitude more effective than the aromatic boronic acids in the inhibition of chymotrypsin. In addition, the synthetic route developed by these workers was instrumental for us in the synthesis of other \( \alpha \)-amino boronic acids used in the preparation of peptide boronic acids for the present study.

**EXPERIMENTAL PROCEDURES**

Bovine chymotrypsin, 49 units mg\(^{-1}\), was obtained from Worthington. Stock solutions were prepared in 1.0 mM HCl and were stored for no longer than three days at 4 °C. Cathepsin G, was a generous gift from Dr. James Travis, University of Georgia, and was stored frozen in 0.10 M pyridine acetate buffer, pH 5.5, and 0.40 M NaCl. Porcine pancreatic elastase and human sputum leukocyte elastase were obtained from Elastin Products, Pacific, MO. Human leukocyte elastase was also prepared by the procedure of Baugh and Travis (1976). Solutions of lyophilized pancreatic elastase were prepared in 0.05 M sodium acetate buffer, pH 5.5, containing 0.40 M NaCl, and were then diluted to 50% glycerol (w/v) and stored at -20 °C. Leukocyte elastase solution (1.0 mg ml\(^{-1}\)) was prepared in 0.05 M sodium acetate buffer, pH 5.5, containing 0.40 M NaCl and stored at -20 °C.

Chymotrypsin and cathepsin G were assayed with the substrate Suc-Ala-Ala-Pro-Val-nitroanilide reported by Nakajima et al. (1979). More sensitive assays of chymotrypsin used Suc-Ala-Ala-Pro-Phe-7-amidino-4-methylcoumarin (Enzyme Systems Products). Pancreatic elastase was assayed with Suc-Ala-Ala-Pro-nitroanilide (Sigma) as a substrate (Bieth et al., 1974). Leukocyte elastase was assayed using MeO-Suc-Ala-Ala-Pro-Val-nitroanilide (Nakajima et al., 1979). Assay solutions contained 1% dimethyl sulfoxide for chymotrypsin, and leukocyte elastase and 5% dimethyl sulfoxide for cathepsin G.

The hydrolysis of p-nitroanilide substrates was monitored at 405 nm on a Cary 219 spectrophotometer. Hydrolysis of 7-amidino-4-methylcoumarin substrates was monitored by excitation at 380 nm and measuring emission at 460 nm on a Perkin-Elmer 650-40 fluorometer.

The concentrations of pancreatic elastase, leukocyte elastase, and chymotrypsin were estimated by the titrimetric procedure described by Morrison (1969) using the most effective peptide boronic acid inhibitor. See data in Fig. 6 for an example. The concentration of cathepsin G was based on weight.

All kinetic constants were determined in 0.10 M sodium phosphate buffer, pH 7.5, containing 0.50 M NaCl at 25 °C. \( K \), values for peptide boronic acids were determined from double reciprocal plots of velocity versus substrate concentration by the method of Lineweaver and Burk. Data in all cases fitted to the best straight lines by the least-squares method. In all cases the inhibitor concentration was at least 5-fold greater than the enzyme concentration. Five or six concentrations of substrate were chosen so that values of reciprocal concentrations were evenly proportioned. The substrate concentration ranges and kinetic constants for substrate hydrolysis are given in Appendix I (Table I). \(^2\)

For reactions in which progressive, time-dependent inhibition was observed, the reactions were initiated by the addition of enzyme and the initial and steady-state velocities were measured. Reaction velocities were measured after 30 and 60 min to establish whether a steady state had been reached. In all cases, no significant difference was observed between double reciprocal plots used to calculate \( k' \), values after 30 and 60 min, and data at 30 min were used to calculate \( k' \). This technique was generally applicable except for chymotrypsin in which a small correction for substrate depletion was necessary. Values of \( k' \), the apparent first-order rate constant for the transition from the initial velocity (\( v_0 \)) to the steady-state velocity (\( v_\infty \)), were determined by the method of Cha (1975) using Equation 1. The spectrophotometer tracings of the reaction progress were extrapolated to the y axis (time = 0) after a steady state was reached and the value of the y intercept, \( r \), was determined. For determining \( k' \), the \( v_0 \) was measured at either 30 or 60 min depending on the inhibitor and enzyme. This method was routinely used for determining values of \( k' \) reported in Appendix I (Table II). The fit of the data to Equation 2 (Cha, 1975) was also determined using the MLAB computer program on the DEC-10 computing system. In Equation 2, \( P \) is the concentration of product at time \( t \), \( P_0 \) is \( P \) at \( t = 0 \).

\[
k' = \frac{1}{r} (v_0 - v_\infty)
\]

\[
P = P_0 + (v_0 - v_\infty)/k' + v_\infty (1 - e^{-kt/k'})
\]

The synthesis of amino boronic acids is described in Appendix II and the preparation of peptide boronic acids is described in Appendix III. In all cases, nuclear magnetic spectra and elemental analyses were consistent with structure.

**RESULTS**

**Synthesis and Properties of Peptide Boronic Acids**—The structures of the \( \alpha \)-amino boronic acid peptides and corresponding diol esters are given in Fig. 2. Compounds were prepared by coupling MeO-Suc-Ala-Ala-Pro-OH to pinacol esters of racemic \( \alpha \)-amino boronic acids. The pinacol esters were converted to diethanolamine esters which were hydrolyzed in the presence of aqueous acid to yield the unprotected peptide boronic acid. In two cases, MeO-Suc-Ala-Ala-Pro-boro-Phe-OH and MeO-Suc-Ala-Ala-Pro-boro-Ala-OH, crystalline diethanolamine derivatives were obtained which allowed separation of the diastereomers. Assignment of the configuration of the boronic acids was based on the activity of crystallization fractions with chymotrypsin and pancreatic elastase assuming that the most active isomer is the "L" form. Previously, this has been shown for the "D" and L forms of Ac-Boro-Phe-OH (Matteson et al., 1981).

The diethanolamine ester of MeO-Suc-Ala-Ala-Pro-boro-Val-OH was not crystalline and therefore the separation of diastereomers was not possible by fractional crystallization. The all-L form of MeO-Suc-Ala-Ala-Pro-boro-Val-OH in

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\(^1\) (R)-Acetamido-2-phenylethyl boronic acid is an analog of N-acetyl-phenylalanine in which the -COOH group is replaced by -B(OH)\(_2\). We have abbreviated this compound and other \( \alpha \)-amino boronic acids by the prefix "boro" and the name of the corresponding amino acid. All amino acids are in the L configuration unless specified. The designation of L for the configuration of \( \alpha \)-amino boronic acids is that of naturally occurring L-amino acids. Other abbreviations used are: Suc, succinyl, MeO-Suc, methoxycycn; Heps, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MS, mass spectrum; Me, methyl; Bz, benzoyl; i-Pr, iso-propyl; Boc, tert-butyloxycarbonyl.

\(^2\) Portions of this paper (including Appendices I–III, Tables I–III, a scheme, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-1514, cite the authors, and include a check or money order for $6.00 per set of photocopies. Full-size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
which the boronic acid was in the form of its pinanediol ester was prepared from H-L-boro-Val-pinanediol which was obtained by a stereospecific synthetic route similar to that described by Matteson et al. (1981).

The stability of MeO-Suc-Ala-Ala-Pro-DL-boro-Val-OH and its pinacol ester have been studied fairly extensively. The pinacol ester is approximately 100-fold less active in the initial inhibition of leucyote elastase than the free acid. However, after incubation in 0.10 M phosphate buffer, pH 7.5, for less than an hour, its inhibitory activity is identical to the free boronic acid. The free boronic acid is stable under these conditions up to 24 h at room temperature. The inhibitory activity of the diethanolamine esters of MeO-Suc-Ala-Ala-Pro-boro-Phe-OH and MeO-Suc-Ala-Ala-Pro-boro-Val-OH and the corresponding free boronic acids were identical even without preincubation.

Results obtained with the pinanediol ester indicate that this ester is also hydrolyzed to yield the free boronic acid. Comparison of the effectiveness of MeO-Suc-Ala-Ala-Pro-L-boro-Val-pinanediol after incubation in phosphate buffer and of MeO-Suc-Ala-Ala-Pro-DL-boro-Val-OH in the inhibition of leucyote elastase were consistent with the hydrolysis of the ester to the free boronic acid. Since similar results were obtained with the other enzymes we have studied, the free boronic acid of MeO-Suc-Ala-Ala-Pro-boro-Val-pinanediol was not characterized, but instead the pinanediol ester was preincubated in 0.10 M sodium phosphate buffer, pH 7.5, for an hour prior to assays in the subsequent studies. The other peptide boronic acids were chemically characterized as the free boronic acids unless specifically stated.

Mechanism of Inhibition by Peptide Boronic Acids—The peptide boronic acids corresponding to the better substrates for chymotrypsin, pancreatic elastase, and leucyote elastase inhibit these enzymes in a progressive, time-dependent manner. We have examined in some detail the mechanism of the inhibition of chymotrypsin by MeO-Suc-Ala-Ala-Pro-boro-Phe-OH, the most effective inhibitor of this enzyme. As shown in Fig. 3, rather than a linear increase in product with time, a decrease in reaction rate is observed in enzyme-initiated assays until a steady state is obtained (middle curve). When the inhibitor is preincubated with enzyme and the reaction is initiated by adding substrate, a progressive increase in reaction rate is observed until the same steady-state velocity as enzyme-initiated assays is obtained. The two progress curves were adequately defined by Equation 2 for time-dependent inhibition. Similar values of $k'$, the rate constant for the first-order transition from initial velocity to steady-state velocity, were obtained regardless of the method of initiating the reaction.

The initial and final velocities of chymotrypsin in the presence of 5.0 nM MeO-Suc-Ala-Ala-Pro-boro-Phe-OH were analyzed by the method of Lineweaver and Burk using data similar to those shown in Fig. 3. As shown in Fig. 4, double reciprocal plots of initial and final velocities intercept the y axis close to the intercept obtained in the absence of inhibitor indicating that inhibition is competitive. $K_i$ values calculated for the initial and final velocities are reported in Table 1.

The results in Figs. 3 and 4 are consistent with the mechanism, Equation 3, for "reversible, slow-binding inhibition" as defined by Williams and Morrison (1979).

$$E + I \stackrel{k_1}{\rightarrow} EI \stackrel{k_1}{\rightarrow} EI^* \quad (3)$$

$$k' = k_0 + k_3 [(I/K_i(initial))/I + I/K_i(initial) + S/K_m] \quad (4)$$

Equation 4 defines $K_i(initial)$, which is $k_0/k_3$, and $K_i(final)$ in terms of rate constants in Equation 3. Equation 5 gives the relationship between $k'$ and the rate constants, $k_0$ and $k_3$ of Equation 4. $K_i(initial)$, inhibitor concentration ($I$), substrate concentration ($S$), and the Michaelis constant ($K_m$) (Schloss et al., 1980).

Kinetic constants $k_0$ and $k_3$ were determined for the reaction of MeO-Suc-Ala-Ala-Pro-boro-Phe-OH with chymotrypsin by two different methods and are compared in Table 1. First, in method 1, $k_3$ was determined experimentally in a manner similar to that described by Schloss et al. (1980). The boronic acid was incubated in a 5-fold molar excess with the enzyme and the mixture was diluted by a large factor in the assay.

![Fig. 3. Effect of MeO-Suc-Ala-Ala-Pro-boro-Phe-OH (I) on hydrolysis of substrate (S) by chymotrypsin (E). Hydrolysis of substrate Suc-Ala-Ala-Pro-Phe-p-nitroanilide was monitored by measuring the change in absorbance at 405 nm with time. In all cases, the final concentrations of S, E, and I were 20.0 μM, 0.76 nM, and 10.0 nM, respectively. I = 0 is the reaction in the absence of inhibitor. For (S + I) + E, the reaction was initiated by adding E to a solution of S and I. (E + I) + S is the reaction in which E and I were preincubated for 5 min and diluted 20-fold into a solution of substrate.](image)

![Fig. 4. Lineweaver-Burk plots for the inhibition of chymotrypsin by MeO-Suc-Ala-Ala-Pro-boro-Phe-OH. Reactions were initiated by adding chymotrypsin to a solution of substrate (S), Suc-Ala-Ala-Pro-Phe-p-nitroanilide, and inhibitor. The final concentration of inhibitor was 5.0 nM and of enzyme was 0.76 nM. Initial velocities were measured immediately after the addition of enzyme, and final velocities were measured after 30 min. Velocities (2,000 × change in absorbance at 405 nm/min) are compared with reactions run in the absence of inhibitor, I = 0.](image)
solution. The increase in enzymatic activity could be monitored by using a sensitive fluorogenic substrate (Fig. 5). Under these conditions, the term in parentheses of Equation 3 is small, and the observed k' is a good approximation of k4. The ratio, k3/k4, was calculated from the values of K(initial) and K(final) using Equation 4.

In method 2, k3 and k4 were determined from the experimental values of k' (Appendix I, Table II) and the graphic solution of Equation 5. A comparison of rate constants obtained by methods 1 and 2 indicates that both gave consistent values of k4 and that there is some variation in k3. We feel method 1 is more accurate since method 2 is dependent on measuring rate constants at high substrate concentrations over a relatively small concentration range.

The deuterium isotope effects on the hydrolysis of Suc-Ala-Ala-Pro-Phe-p-nitroanilide and on the binding reactions of MeO-Suc-Ala-Ala-Pro-boro-Phe-OH to chymotrypsin were determined (Table I). For substrate hydrolysis, values of K were similar in both H2O and D2O while a large isotope effect, approximately 3-fold, was observed for kcat. Comparison of the isotope effect on inhibitor binding demonstrates only small differences in K(initial) and K(final).

Values of k3 and k4 determined by the graphic solution of Equation 5 (method 2) were very similar. In method 1, again, almost identical values of k4 were obtained but a larger deviation, approximately 80%, was observed in k3. Although our methods of analysis are not sensitive to small changes in rate, an isotope effect of the magnitude observed in kcat for substrate hydrolysis was not observed.

<table>
<thead>
<tr>
<th>H2O</th>
<th>D2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcat (µM)</td>
<td>24.7 ± 4.4</td>
</tr>
<tr>
<td>kcat (s⁻¹)</td>
<td>26.0 ± 4.2</td>
</tr>
<tr>
<td>K(initial) (nM)</td>
<td>3.4 ± 1.1</td>
</tr>
<tr>
<td>K(final) (nM)</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>k3/k4 Method 1</td>
<td>20.2</td>
</tr>
<tr>
<td>k3 (s⁻¹)</td>
<td>0.020</td>
</tr>
<tr>
<td>k4 (s⁻¹)</td>
<td>0.0010</td>
</tr>
</tbody>
</table>

* Kcat and kcat values were determined by the method of Lineweaver and Burk for the hydrolysis of MeO-Suc-Ala-Ala-Pro-Phe-p-nitroanilide. In both H2O and D2O, the concentration range of substrate was 0.050–0.50 nM and the concentrations of enzyme were 0.78 and 2.4 nM, respectively.

For H2O, the reported value is the average of 5 determinations (S.D. ± 0.00015 s⁻¹). Using the relationship in Equation 4 (K(initial)/K(final) = 1/k4/kcat) by plotting the observed values of k' (Appendix I, Tables II and Tables III) versus the term in the parentheses of Equation 5. The plot was linear over a substrate range of 0.10–0.50 mM for reactions run both in H2O and D2O.

Finally, the stoichiometry of binding of MeO-Suc-Ala-Ala-Pro-boro-Phe-OH to chymotrypsin was determined as shown in Fig. 6. The protease was incubated with less than stoichiometric amounts of inhibitor, and per cent activity was determined from controls run in the absence of inhibitor. A linear relationship was obtained between per cent activity and inhibitor concentration over most of the range of inhibitor concentrations. The concentration of chymotrypsin, based on the dry weight of protein and molecular weight of 25,100 is 597 nM. The deviation we have observed is well within experimental error and leaves little doubt that MeO-Suc-Ala-Ala-Pro-boro-Phe-OH forms a 1:1 complex with chymotrypsin.
Inhibition of Serine Proteases

Specificity of Peptide Boronic Acids—Peptide boronic acids were prepared which correspond to the structure of the preferred sequence of substrates for chymotrypsin, cathepsin G, pancreatic elastase, and leukocyte elastase by varying the residue in the P$_2$ site. Chymotrypsin and cathepsin G prefer an aromatic residue in the P$_2$ site while pancreatic elastase and leukocyte elastase prefer Ala and Val, respectively. Smaller differences exist in the specificity of this group of enzymes for residues in the P$_2$–P$_1$ sites and MeO-Suc-Ala-Ala-Ala-Pro- is one of the best sequences for all the proteases.

The kinetic constants for the reactivities of the proteases with peptide boronic acids are shown in Table II. $K_i$ values shown are average values reported with standard deviations for repetitive measurements. Inhibitor concentrations used in measuring $K_i$ values are also given in Table II while substrates, substrate concentration ranges, protease concentrations, and kinetic constants obtained for substrate hydrolysis are given in Appendix I (Table I). All kinetic constants for substrate hydrolysis were consistent with those reported in the literature.

In all cases, inhibition was competitive and both final and initial $K_i$ values are reported for all reactions of inhibitors in which slow-binding inhibition was observed. The possibility that other compounds in Table II exhibit time-dependent inhibition upon prolonged incubation with the enzyme exist, but it was not observed during the 3–4-min assay period over the concentration ranges of substrates and inhibitor we have used. Therefore, any transformation into a complex more stable than that reflected by the $K_i$ for initial complex would have to be a considerably slower process than observed with the time-dependent inhibitors we report.

For chymotrypsin, MeO-Suc-Ala-Ala-Pro-boro-Phe-OH exhibited slow-binding inhibition with an initial $K_i$ of 3.4 nM and a final or steady-state $K_i$ of 0.16 nM. All other compounds appeared to be simple competitive inhibitors of this enzyme. The affinity of MeO-Suc-Ala-Ala-Pro-boro-Phe-OH for chymotrypsin is at least 50-fold greater than the corresponding boro-Ala-OH peptide. Comparison of Ac-boro-Phe-OH and Me-O-Suc-Ala-Ala-Pro-boro-Phe-OH illustrates the extent to which the peptide portion contributes to overall binding affinity. The kinetic constant for Ac-boro-Phe-OH in Table II is that of Matteson et al. (1981) who report that it is a simple competitive inhibitor.

Comparison of the affinity of cathepsin G and chymotrypsin for the peptide boronic acids indicates that binding has a similar dependence on the nature of the residue in the P$_2$ site. However, in contrast to chymotrypsin and the other serine proteases in Table II, no time-dependent inhibition was observed for cathepsin G. Time-dependent inhibition could not be detected for even the most effective inhibitor, MeO-Suc-Ala-Ala-Pro-boro-Phe-OH, after a 30-min incubation with the enzyme.

For pancreatic elastase, both MeO-Suc-Ala-Ala-Pro-boro-Val-OH and MeO-Suc-Ala-Ala-Pro-boro-Ala-OH are similar in effectiveness, exhibiting steady-state $K_i$ values in the 0.2–0.3 nM range. Differences between these two inhibitors are reflected in a 2-fold lower $K_i$ for initial binding of the boro-Ala-OH peptide. For leukocyte elastase, MeO-Suc-Ala-Ala-Pro-boro-Val-OH is the only inhibitor which exhibited slow-binding inhibition and it is several orders of magnitude more effective than the corresponding boro-Ala-OH peptide. The boro-Phe-OH peptide was considerably less active for both elastolytic enzymes. Individual kinetic constants, $k_a$ and $k_d$, for the slow-binding inhibitors of the elastolytic enzymes are given in Appendix I (Table III). It should be noted that there

### Table II

**Inhibition of serine proteases**

<table>
<thead>
<tr>
<th>Protease</th>
<th>Inhibitor (R-MeO-Suc-Ala-Ala-Pro-)</th>
<th>$K_i$</th>
<th>Concentration of inhibitor</th>
<th>Number of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>nM</td>
<td>nM</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>Ac-Boro-Phe-OH$^b$</td>
<td>2,106</td>
<td>11.0 ± 2.3</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>R-Boro-Phe-OH</td>
<td>3.4 ± 1.1</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>R-Boro-Val-OH</td>
<td>1,200 ± 50</td>
<td>5,000</td>
<td>10,000</td>
</tr>
<tr>
<td></td>
<td>R-Boro-Ala-OH</td>
<td>9,100 ± 900</td>
<td>100</td>
<td>25,000</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>R-Boro-Phe-OH</td>
<td>21.0 ± 0.2</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R-Boro-Val-OH</td>
<td>500 ± 80</td>
<td>400</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R-Boro-Ala-OH</td>
<td>74,000 ± 14,000</td>
<td>100,000</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>112,000 ± 2,000</td>
<td>150,000</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pancreatic elastase$^d$</td>
<td>R-Boro-Phe-OH</td>
<td>270 ± 50</td>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R-Boro-Val-OH</td>
<td>30 ± 13</td>
<td>0.25 ± 0.02</td>
<td>10</td>
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<tr>
<td></td>
<td>R-Boro-Ala-OH</td>
<td>18.2 ± 2.5</td>
<td>0.32 ± 0.04</td>
<td>10</td>
</tr>
<tr>
<td>leukocyte elastase$^e$</td>
<td>R-Boro-Phe-OH</td>
<td>350 ± 80</td>
<td>2,500</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R-Boro-Val-OH</td>
<td>15 ± 6</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R-Boro-Ala-OH</td>
<td>79 ± 25</td>
<td>100</td>
<td>3</td>
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</table>

$^a$ The concentrations of inhibitor and number of determinations are reported. The enzyme concentrations, substrates, substrate concentration ranges, and values of $K_m$ and $k_{cat}$ are reported in Appendix I (Table I).

$^b$ The $K_i$ for Ac-boro-Phe-OH was reported by Matteson et al. (1981).

$^c$ The rate constants, $k_a$ and $k_d$, for the $E' - EI'$ transition are reported in Table I.

$^d$ For pancreatic elastase, the respective rate constants, $k_a$ and $k_d$, for the boro-Val-OH peptide were 0.026 and 0.00093 s$^{-1}$ and for the boro-Ala-OH peptide were 0.018 and 0.00941 s$^{-1}$ for leukocyte elastase and the Boro-Val-OH peptide they were 0.0092 and 0.00038 s$^{-1}$. See Appendix I, Tables II and III.

$^e$ The reported kinetic constants were determined with elastase isolated from sputum. Similar values were obtained for elastase isolated by the procedure of Baugh and Travis (1976) for MeO-Suc-Ala-Ala-Pro-boro-Val-OH, i.e. $K_i$(initial) 16 ± 6 nM and $K_i$(final) 0.38 ± 0.08 nM.
is not much more than a 2-fold difference in values of \( k_b \) or in values of \( k_a \) for the elastases and chymotrypsin (Table 1). Considerable refinement of kinetic measurements would be required to ascertain the effect that structure has on the small differences in rate constants.

**DISCUSSION**

Peptide boronic acids are a unique group of effective protease inhibitors which exhibit reversible, slow-binding inhibition. The property of slow-binding inhibition, however, is limited to those inhibitors which satisfy the primary specificity requirements for the protease and in this study were analogs of the more effective substrates. Slow-binding inhibition has been observed for inhibitors of a number of enzymes (Rich and Sun, 1980; Baici and Gyger-Marazzi, 1982; Morrison, 1982; Schloss and Cleland, 1982), but it has not been reported for a synthetic, serine protease inhibitor. Very recently, slow-binding inhibition was reported for the serine protease neutrophil elastase by eglin, a 70-residue polypeptide inhibitor isolated from leech (Baici and Seemuller, 1984). In many cases, slow-binding inhibition is associated with inhibitors which act as transition-state analogs (Stark and Bartlett, 1983) or more generally, as reaction-intermediate analogs (Schloss and Cleland, 1982).

There is little doubt the peptide boronic acids, which are the more effective protease inhibitors, form a transition state-like complex with the active site serine of the protease. The affinity of the peptide boronic acid inhibitor for chymotrypsin exceeds that of the corresponding substrate by at least 4 orders of magnitude and other peptide analogs by at least several orders of magnitude. For example, chymostatin, a bacterial peptide terminating in the aldehyde analog of phenylalanine, has an IC\(_50\) of 2.5 \( \times \) 10\(^{-7} \) M for chymotrypsin (Umezawa and Aoyagi, 1977) while the final \( K_f \) for MeO-Suc-Ala-Ala-Pro-boro-Phe-OH is 1.6 \( \times \) 10\(^{-10} \) M. It would be difficult to explain the high affinity of the peptide boronic acids without the contribution of the tetrahedral complex between the active site serine and the boronic acid moiety. Furthermore, a tetrahedral complex has been observed in the crystallographic study of the bacterial serine protease subtilisin and phenylenyhane boronic acid (Matthews et al., 1975). The affinity of phenylenyhane boronic acid for subtilisin is considerably lower than even the less effective peptide boronic acids examined in this study.

The progressive, time-dependent inhibition we have observed is not readily explained on a molecular basis. One can speculate that initial binding is the formation of a ground-state complex with the enzyme and that the slower kinetic stage of the reaction involves the formation of a transition state-like tetrahedral complex of the boronic acid and the active-site serine. An analogy can be drawn between this mechanism and the mechanism for substrate hydrolysis leading to the formation of an acyl-enzyme (Fig. 1). Formation of the acyl enzyme is the rate-limiting step in the hydrolysis of most amide substrates, and a significant isotope effect is observed on \( k_{cat} \) due to the associated proton transfer (Stein, 1983). Nakatani et al. (1975) have shown that binding of phenyl boronic acid to subtilisin occurs by a similar two-step process. The first is the formation of a complex between the enzyme and the trigonal boronic acid and the second is the formation of a tetrahedral adduct with the active site serine. Like substrate hydrolysis, a deuteron isotope effect was observed for the latter step. It should be noted that the transition these workers have observed is 5 orders of magnitude faster than those we have observed and would not be considered slow-binding inhibition (Williams and Morrison, 1979). Therefore, comparable deuteron isotope effects on \( k_b \) for peptide boronic acid binding and on \( k_{cat} \) for substrate hydrolysis would provide evidence for this mechanism. This, however, was not observed. Even though our measurements of the rate constant for the formation of the more stable complex, \( k_b \), are error prone, it is clear that the 3-fold isotope effect for \( k_{cat} \) is not observed for \( k_b \).

Considering both the relative high effectiveness of peptide boronic acids over other peptide analogs and the absence of a definitive deuteron isotope effect on \( k_b \), we feel that all compounds probably form a tetrahedral complex with the active site serine and the slow binding phase of the inhibitor-enzyme reaction is due to a conformational change of the enzyme to optimize binding similar to that expected for binding of substrate in the transition state. Conformational changes have been observed in the crystal structure of the complex between Streptomyces griseus protease A and a tetrapeptide aldehyde inhibitor which also forms a transition state-like complex with the active-site serine (Brayer et al., 1979). However, additional studies are required to provide definitive proof of the origin of slow-binding inhibition observed for the peptide boronic acids.

Some of the structural requirements for boronic acids to exhibit slow-binding inhibition of chymotrypsin have been determined. First, binding in the primary specificity site (P1) alone, as is the case for the inhibition of chymotrypsin by Ac-boro-Phe-OH (Matteson et al., 1981), will not give slow-binding inhibition. For peptide boronic acids, the specificity requirement of the enzyme in the P1 site must be met for progressive inhibition since analogs of MeO-Suc-Ala-Ala-Pro-boro-Phe-OH containing Boro-Ala-OH and Boro-Val-OH are simple competitive inhibitors. The stereochemistry of the P1 boronoaminic acid is also important since MeO-Su-z-Ala-Ala-Pro-D-boro-Phe-OH behaves as a simple competitive inhibitor. Although this inhibitor is almost two orders of magnitude less effective than the corresponding L isomer, it is still a very effective inhibitor.

All peptide boronic acids which correspond to the sequence of the better substrates for individual proteases were by far the most effective inhibitors. However, inhibitor effectiveness was not entirely predicted from the substrate specificity of the protease. Zimmerman and Ashe (1977), Nakajima et al. (1979), and McRae et al. (1980) have studied the specificity of serine proteases by varying the P1 residue of N-acyl peptide-p-nitroanilide and amide substrates containing P, P-Ala, P-Ala, and P-Pro. These studies have shown that substrates containing a Phe in the P1 site are selective substrates for chymotrypsin and cathepsin G, and those with Ala and Val in the P1 sites are selective substrates for leukocyte and pancreatic elastase. The chymotrypsin substrates are not hydrolyzed by the elastases, and the elastase substrates are not hydrolyzed by chymotrypsin and cathepsin G. Comparing the two elastolytic enzymes, leukocyte elastase exhibits an approximate 10-fold preference for the Val substrate, and pancreatic elastase has a similar preference for the Ala substrate. MeO-Suc-Ala-Ala-Pro-boro-Phe-OH was 4 orders of magnitude more effective in the inhibition of chymotrypsin and cathepsin G than reagents with boro-Val and boro-Ala in the P1 site. MeO-Suc-Ala-Ala-Pro-boro-Val-OH is the most effective inhibitor for leukocyte elastase and the boro-Val and bor-Ape peptides were 100 and 500-fold less effective. For pancreatic elastase, MeO-Suc-Ala-Ala-Pro-boro-Ala-OH and MeO-Suc-Ala-Ala-Pro-boro-Val-OH are similar in effectiveness and are 3 orders of magnitude more effective than the corresponding Boroph peptides. It should be noted that the Boroph peptide is still an effective inhibitor of both elastases and this clearly would
not have been predicted from the enzyme’s substrate specificities.

The high affinity of peptide boronic acids \( (K_v = 0.2-20 \text{ nM}) \) and their selectivity for particular proteolytic enzymes should make them valuable for study of the physiological roles of cathepsin G and leukocyte elastase. In addition, they may be useful for the treatment of diseases in which aberrant proteolysis has been implicated such as in emphysema and sepsis (Hardie et al., 1984).³

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REFERENCES


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**Inhibition of Serine Proteases**

**Table I. Kinetic Constants for Selective Inhibition of Serine Proteases**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Iodoacetamide</th>
<th>Iodoacetic Acid</th>
<th>iodoacetamide</th>
<th>iodoacetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chymotrypsin</td>
<td>0.003</td>
<td>0.001</td>
<td>0.004</td>
<td>0.002</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.002</td>
<td>0.001</td>
<td>0.003</td>
<td>0.002</td>
</tr>
</tbody>
</table>

**Synthetic Scheme for α-Aminoboronic Acids**

1. **Step 1:**
   - **Reagents:**
     - Ethylboronic acid
     - Benzene
   - **Products:**
     - Ethylboronic acid benzene

2. **Step 2:**
   - **Reagents:**
     - Benzoic acid
     - Water
   - **Products:**
     - Benzyl benzoate

3. **Step 3:**
   - **Reagents:**
     - Sodium thiosulfate
     - Sodium chloride
   - **Products:**
     - Sodium thiosulfate sodium chloride

4. **Step 4:**
   - **Reagents:**
     - Sodium carbonate
     - Sodium chloride
   - **Products:**
     - Sodium carbonate sodium chloride

**General Methods:**

- **Hypothalamic Neurons:**
  - Neurons were isolated from a 5- to 6-week-old rat
  - Neurons were loaded with Dye using a patch-clamp technique

- **Confocal Imaging:**
  - Confocal images were acquired using a Zeiss LSM 510 confocal microscope
  - Images were processed using ImageJ software
Inhibition of Serine Proteases

15114

Inhibition of Serine Proteases

17.60; B. 3.24. Pound: C. 51.541; l.562; and ad 8. 2.111. mal. Old.

For C. II W 0 8: C. 51.661; H, 7.501. N. 13.701; and 8. 2.111.

References


