X-ray Crystallographic Study of Boronic Acid Adducts with Subtilisin BPN' (Novo)

A MODEL FOR THE CATALYTIC TRANSITION STATE*

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SUMMARY

We have studied the structures of adducts formed between subtilisin BPN' and both benzeneboronic acid and 2-phenylethaneboronic acid by x-ray diffraction techniques. Electron density and difference maps at 2.5 A resolution were computed with phases calculated from a partially refined structure of the native enzyme (R = 0.23 at 2.0 A). Both adducts contain a covalent bond between Oγ of the catalytic Ser-221 and the inhibitor boron atom. The boron atom is coordinated tetrahedrally, with one of the two additional boronic acid oxygen atoms lying in the "oxyanion hole" and the other at the leaving group site identified in previous studies (ROBERTUS, J. D., KRANTZ, J. P., ALDEN, R. A., AND BIRKTOFT, J. J. (1972) Biochemistry 11, 4293-4303). Moreover, the previously postulated structure of the tetrahedral intermediate for substrate hydrolysis is isosteric with these boronic acid adducts, which can therefore be considered good models for the transition state complex (KOHLER, K. K., AND LIENHARD, G. E. (1972) Biochemistry 10, 2477-2483). These observations further support the suggestion that an important contribution to stabilization of this transition state complex, relative to both the Michaelis complex and the acyl intermediate, occurs as a consequence of hydrogen bond donation to the substrate carbonyl oxygen atom from the side chain amido group of Asn-155 and from the backbone amido group of Ser-221.

In previous communications from our laboratory we have discussed in detail the binding to subtilisin BPN' (Novo) of various polypeptides and various chloromethyl ketone analogs of the acylating portion of good substrates (2, 3). The binding geometries of the inhibitor-enzyme complexes were arrived at by interpreting difference Fourier maps computed with multiple isomorphous replacement phases from phenylmethanesulfonfyl-

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1 Subtilisin's BPN' and Novo are identical species (1) which we shall refer to simply as "subtilisin."

subtilisin (4). At that time we argued that the structure of the Michaelis complex between the enzyme and true polypeptide substrates could be deduced from these observations (2, 3), and further model-building experiments then led to proposed structures for the two other known catalytic intermediates (3): a tetrahedral addition compound and an acyl enzyme. In the case of the tetrahedral intermediate, one particular configuration about the carbonyl carbon of the susceptible peptide bond seemed especially attractive in that it could be stabilized by two hydrogen bonds from donors on the enzyme to the carbonyl oxygen, now bearing a formal negative charge, while simultaneously positioning the leaving group to accept a proton from His-64. While this model offered a convincing example of transition state stabilization due to specific interaction between substrate and enzyme, its ultimate justification awaited more direct experimental evidence. In this paper we present further support for the above stereochemical picture of the catalytic mechanism based upon the structure of adducts between subtilisin and both benzeneboronic acid and 2-phenylethaneboronic acid.

Recently Lienhard and co-workers have studied the strong inhibition of α-chymotrypsin by boronic acid derivatives containing an aromatic side chain which is presumably inserted into the specificity pocket adjacent to the catalytic site of the enzyme (5, 6). This inhibition is characterized by an unusually large binding constant and a pH dependence which together appear to rule out simple noncovalent association. These authors present a variety of possible structures for the boronic acid-enzyme complex and suggest that the most probable ones involve a tetrahedral boron covalently attached to either the Oγ of Ser-195 or to the Ne2 of His-57, or to both. If (I), the most probable structure, were correct it might provide an approximate analog for the tetrahedral intermediate and thus have a geometry close to that of the transition state for acylation and deacylation of the catalytic serine. Replacement of the oxyanion in the tetrahedral intermediate by a hydroxyl group in the boronic acid should not severely affect the ability of the bound group to accept hydrogen bonds from backbone and side chain atoms surrounding the oxyanion hole.
Lindquist and Terry (7) have reported on the inhibition of subtilisin Carlsberg by boronic acids and reached similar conclusions to those reported earlier for α-chymotrypsin by Lienhard's group. It had previously been shown that the geometries of the catalytic and binding sites of subtilisin and α-chymotrypsin are sterically very similar at present levels of resolution (2), and that subtilisins Carlsberg and BPN' (Novo) should be structurally homologous (4). Thus our conclusions with respect to subtilisin enzymes and to chymotrypsin as well.

MATERIALS AND METHODS

Benzenboronic acid was obtained from the Aldrich Chemical Co. 2-Phenyltheneboronic acid was a gift from Dr. Lienhard (Dartmouth Medical School). Subtilisin was purchased from the Novo Enzyme Corp., and purified and crystallized at pH 5.9 as previously described (2).

The boronic acids under investigation exhibit maximum binding near pH 7.5 (5, 7, 8). We have found that crystals of subtilisin can easily be raised to this pH with no apparent deterioration. Crystals suitable for x-ray diffraction measurements were transferred to a solution of 2.1 M ammonium sulfate buffered at pH 7.5 with 0.05 M sodium phosphate. After soaking at 4°C for a period of 1 week some of the pH 7.5 crystals were transferred to a fresh solution of the same composition and pH, but saturated with one or the other boronic acid, and allowed to soak at 4°C for 10-14 days longer.

Data crystals of native subtilisin at pH 7.0 and of both boronic acid adducts were mounted as usual in thin-walled borosilicate glass capillaries, with the c axis of the reciprocal net coincident with the φ axis of a computer-controlled four-circle Hilger and Watts diffractometer which was used for all intensity measurements. Twelve reflections with 2θ greater than 25° were carefully centered and the final angular settings for these reflections were used in the least squares calculation of lattice parameters. The lattice parameters were within 0.2% of those determined for native subtilisin at pH 5.9. Intensities were collected using an 11-point scan across the tops of the peaks at 0.01° increments. Integrated reflection intensities were assumed to be proportional to the maximum sum of four contiguous individual step counts. Background corrections were assumed to depend only upon the Bragg angle. An empirical absorption correction depending only upon the φ setting was applied; typically it varied about 30% between extremes. Two symmetry equivalent sets of 2.5 A data were collected (hk0 and hkl, space group C2). A single crystal was used for all intensity measurements of the parent and each derivative. Standard reference reflections were monitored after every 100 measurements and typically showed intensity decreases of 15 to 20% at the termination of data collection.

Difference Fourier maps depicting the bound boronic acid inhibitors and revealing any induced changes in enzyme geometry were calculated using (Fobs - Fcalc) as coefficients. In this notation $F_{\text{calc}}$ refers to the observed structure amplitudes for an enzyme-inhibitor complex and $F_{\text{obs}}$ to the observed structure amplitudes for native subtilisin at pH 7.5. In order to avoid overlapping any structural changes that might occur simply as a result of changing the pH of the native enzyme from 5.9 to 7.5, a difference Fourier map with coefficients $(F_{\text{obs}} - F_{\text{calc}})$ was also calculated. For both maps phases were derived by structure factor calculations of our current model for the native structure at pH 5.9. This model has an R factor of 0.23 at 2.0 A resolution after 11 cycles of unconstrained difference-Fourier refinement in which positional parameters and individual isotropic temperature factors have been allowed to vary for all nonhydrogen atoms in the structure. The refinement procedure was essentially that described by Freer et al. (9) and fully discussed elsewhere. All maps were calculated on a $1 \times 0.7 \times 1$ A grid and plotted on a 2 cm/A scale. The root-mean-square difference densities, σ, were calculated over all grid points in the unit cell. The value of σ was the same, 0.035 e/A³, for both boronic acid difference maps, and was 0.025 e/A³ for the $(F_{\text{obs}} - F_{\text{calc}})$ map.

RESULTS

The difference map calculated with coefficients $(F_{\text{obs}} - F_{\text{calc}})$ showed that for the purposes of this communication we can regard the structures of native subtilisin as identical at the two pH values. However, a small but significant pH-induced movement of the catalytic Asp-32 side chain, about 0.1 to 0.2 A, is in fact observed. An interpretation of this movement, together with a reexamination of the catalytic serine side chain's orientation in the native enzyme, will be presented in a later paper.

Both boronic acid difference maps gave sharp, clearly defined representations of the inhibitor electron density at the active site of the enzyme at pH 7.5. The maps show that both inhibitors are bound to the enzyme in the same manner.

As a means of establishing approximate occupancies for the boronic acid inhibitors, the appropriate region of positive difference density in each map was integrated and compared to that obtained for subtilisin inhibited with the chloromethyl ketone Phe-Ala-Lys-CH₂Cl. The same calculated structure factor phases were used to compute both of the boronic acid maps and the chloromethyl ketone map. The chloromethyl ketone derivative was assumed to be 100% occupied since incubation of the enzyme with this inhibitor prior to crystallization results in complete loss of activity. This comparison showed the primary binding site to be fully occupied for both boronic acid inhibitors. In addition a secondary site that was at least 40% occupied was observed for 2-phenyletheneboronic acid.

Because the benzenboronic acid map is less complicated than that of 2-phenyletheneboronic acid, although the most important features of the two yield identical conclusions, we shall describe it in greater detail.

Our interpretation of the benzenboronic acid difference Fourier is shown in Fig. 1, where a stick model of the inhibitor is superimposed upon the difference electron density contours. The orientation of the inhibitor molecule relative to the enzyme's catalytic residues is shown in Fig. 2. A list of inhibitor coordinates appears in Table I.

The boron atom forms a covalently bonded tetrahedral adduct with Oγ of the catalytic site Ser-221. One hydroxyl group (O1' in Table I) of the boronic acid is adjacent to the catalytic histidine side chain 2.5 A from Nε2. This is the expected distance for a hydrogen bond between the two, but the angle of any such hydrogen bond would be rather distorted. We shall discuss this peculiarity in a subsequent communication. The second inhibitor hydroxyl group (O2' in Table I) is situated in the oxyanion hole (8) and can thus hydrogen bond to both the side chain amide of Asn-155 and to the backbone amido group of Ser-221. The lengths of these hydrogen bonds are 2.5 and 3.0 A.

A broad positive region adjacent to the side chain of Asn-155 reaches contour levels of 5σ in both boronic acid difference maps. This feature, in conjunction with the presence of a weakly negative region nearby, suggests that slight repositioning of the Asn-155 side chain and marked reduction in its apparent temperature factor has occurred as a consequence of inhibitor binding; this is consistent with the formation of a strong hydrogen bond between the boron hydroxyl group O2' and Nε2 of Asn-155. The repositioning is best described as a 15° rotation about the Cβ-Cγ bond of Asn-155. It is significant that this change in position results in a nearly linear hydrogen bond between the side chain nitrogen of Asn-155 and the boronic acid hydroxyl group.
in the oxanion hole. This interpretation also offers the following explanation for regions of negative density, at the 3σ level in both boronic acid difference maps, coincident with the position of solvent molecule W203 in the native structure. In the native enzyme at pH 7.5 a weak hydrogen bond exists between the side chain nitrogen of Asn-155 and W203. In the boronic acid complexes, however, the induced side chain rotation described above lengthens and thus weakens this hydrogen bond, thereby disordering W203.

Several other features of the difference maps can also be interpreted in terms of partial or complete disordering of solvent on the enzyme surface accompanying inhibitor binding. Such effects would provide a favorable entropy contribution to the free energy of binding for both substrates and inhibitors. Steric repulsions attributable to introduction of an inhibitor hydroxyl group near the imidazole side chain of His-64 force displacement of W202 and W303 from the active site region. In the native enzyme, solvent molecule W202 is tightly bound near the postulated position for the leaving group. (In fact, it now seems likely that W202 is a large ion and not a water molecule as originally assumed.) Displacement of these solvent molecules by the inhibitor hydroxyl groups somewhat complicates interpretation of the difference maps owing to partial cancellation of negative and positive density. In particular, the density representing boronic acid oxygen O1' is relatively weak owing to displacement of W202, but this did not cause any ambiguity in its positioning.

Additional disordering of solvent near the extended backbone segment that binds the inhibitor phenyl group is indicated by negative peaks of height 4 to 5σ at the positions of W88, W201, and W205 in the native enzyme. Evidently, however, these solvent molecules are not completely disordered since 2Fo - Fo maps show residual positive density, implying partial occupancy at these sites in the boronic acid-enzyme complexes.

The location of the aromatic groups of both boronic acid inhibitors is thoroughly consistent with what is already known about the geometry of substrate binding to subtilisin. Subtilisin

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**Table I**

|  | X  | Y  | Z  |
|---|----|----|----|
| Cα(221) | 22.0 | 27.7 | 21.3 |
| Oγ(221) | 21.1 | 28.0 | 20.3 |
| B | 19.9 | 27.9 | 20.9 |
| O1' | 19.7 | 26.8 | 21.7 |
| O2' | 19.0 | 27.7 | 19.7 |
| Ca' | 19.0 | 29.2 | 21.5 |
| Cα1' | 17.6 | 29.3 | 21.3 |
| Cα2' | 19.7 | 30.2 | 22.2 |
| Cγ1' | 17.0 | 30.5 | 21.8 |
| Cγ2' | 19.1 | 31.3 | 22.7 |
| Cα' | 17.7 | 31.4 | 22.5 |

* X, Y, and Z are measured in Angstroms in the Cartesian coordinate system defined by Alden et al. (1971) (10).
catalyzes hydrolysis of peptide bonds in which the carbonyl group is donated by amino acids with large hydrophobic side chains. The binding locus, $S_1$, for the hydrophobic side chain is a well-defined crevice that is apparently important in correctly positioning the susceptible peptide bond relative to the catalytic residues in order to facilitate hydrolysis. One side of this crevice is comprised of the planar peptide groups of the extended backbone segment Ser-125-Leu-126-Gly-127. The plane of the benzeneboronic acid's phenyl ring lies almost parallel to this backbone segment at an average distance of 3.5 Å. On the opposite side of the crevice, this phenyl ring is in van der Waals contact with the methyl groups on the side chain of Ala-152 and Thr-220 and the $\alpha$ carbon of Gly-154. Close contact also occurs between the inhibitor phenyl ring and the side chain of Asn-155. W 308 is ejected from its position in the $S_1$ specificity pocket and consequently attenuates the inhibitor side chain density near $C'$. The single feature that remains to be interpreted in the benzeneboronic acid difference map is a negative region of density at 5 Å located adjacent to the position of the Ser-221 Oy in the tetrahedral adduct. This is attributable to a slight relocation, by about 1 Å, of the Ser-221 Oy from its position in the native enzyme to a new site consistent with covalent attachment to the boron atom of the inhibitor. However, according to this interpretation, the required rotation of the Ser-221 side chain about the Ca—Cβ bond upon going from native subtilisin to the boronic acid complex is only $+40^\circ$, much less than the $-130^\circ$ rotation (2.1 Å displacement) expected on the basis of the orientation of this side chain previously thought to exist in the native enzyme. We believe this observation is highly significant, and its implication for a revised view of the native structure of the charge relay system in subtilisin, together with further evidence bearing on this point, will be discussed in another paper.2

In order to test further the above interpretations, four cycles of unconstrained difference-Fourier refinement were carried out using the 2.5 Å data collected for the benzeneboronic acid-subtilisin complex. Positional parameters and an isotropic thermal parameter were allowed to vary for each atom in the boronic acid inhibitor, for each atom in residues 155 and 221, and for all solvent molecules near the inhibitor. Positional and thermal parameters for the remaining atoms were taken from the R = 0.23 structure of the native enzyme at pH 5.9, except that solvent molecules W 202, W 303, and W 308 were removed from the structure factor calculation because they are displaced as a consequence of inhibitor binding.

After refinement no atom had moved more than 0.4 Å. The Ser-221 Ca—Cβ and Cβ—Oy bond lengths were 1.8 and 1.3 Å, in adequate agreement with expected values of 1.5 and 1.4 Å. The B—Oy distance is 1.3 Å, as compared with an expected distance of 1.5 Å. Isotropic temperature factors for Ca, Cβ, and Oy of Ser-221 and for the inhibitor boron are 10, 6, 12, and 8 Å², respectively. Thus unconstrained difference-Fourier refinement does not cause any physically unreasonable drift in the parameters, and so we conclude that covalent attachment of the boron atom to the catalytic serine is defined unambiguously.

We turn now to a discussion of special features found in the difference map calculated for the 2-phenylethaneboronic acid adduct.

The $-\text{B(OH)}_2$ functional group of the 2-phenylethaneboronic acid inhibitor is attached to the active site Ser-221 in a manner virtually identical with that described above for benzeneboronic acid (see Fig. 3). However, in contrast to benzeneboronic acid, 2-phenylethaneboronic acid is also strongly bound to a second site on the enzyme (about 40% occupancy) in close proximity to the region believed to bind the leaving group residue $P_1'$ in a true polypeptide substrate (3). In addition, certain minor differences in binding geometry are seen in the $S_1$ specificity crevice region. They are interpreted as arising from differences in the stereochemistry of the two boronic acid inhibitors.

The aromatic side chain of 2-phenylethaneboronic acid is thrust much deeper into the $S_1$ specificity crevice than is that of benzeneboronic acid. Negative density running along the inside of this crevice from the carbonyl oxygen of Leu-126 to the amido nitrogen of Gly-127 is matched by equivalent positive density on the opposite side of the 125–127 backbone segment. We interpret this as an expansion of the crevice by perhaps as much as 0.5 Å that is induced by the inhibitor; without this expansion excessively short aromatic ring to backbone distances of approximately 3.0 Å would exist. The same phenomenon was also noted in the case of certain polypeptides and chloromethyl ketone inhibitors of subtilisin (2, 3). At other points within the crevice the inhibitor side chain makes additional van der Waals contacts similar to those described above for benzeneboronic acid.

In the 2-phenylethaneboronic acid molecule bound at the primary site, the plane defined by B, Cβ', and Cγ' is nearly perpendicular to the plane of the benzene ring. Sterically this is the most stable conformation in that it minimizes nonbonded interactions between the phenyl group and hydrogen atoms attached to Cγ'. In a real polypeptide substrate however, one Cα' hydrogen on the boronic acid inhibitor would be replaced by the amido nitrogen of the substrate residue $P_1$. Then, if the present conformation were conserved, this nitrogen and any trailing

FIG. 3. The active site region of subtilisin with 2-phenylethaneboronic acid covalently bound to Oy of Ser 221. A noncovalently bound molecule of this inhibitor is shown occupying a secondary site on the leaving group side of the enzyme surface.
residues would come into collision with the enzyme surface at Asn-185. However, as a result of previous inhibitor studies it was concluded this nitrogen would be positioned in such a way that it could hydrogen bond to the carbonyl oxygen of Ser-125 in the tetrahedral intermediate and in the acyl enzyme (3). Therefore this conformation cannot correspond to that of a real substrate. In fact the expected conformation can be readily achieved by simultaneous rotations about the B—Ca', Ca'C—Ca'3, and Ca'2—Ca' bonds and a slight repositioning of the B(OH)2 group (less than 0.2 Å) in the active site while still maintaining the favorable hydrogen bonding at both hydroxyl groups, and while still maintaining the position of the aromatic group in the S1 crevice.

Perturbations of the structured solvent concomitant with binding of 2-phenylethaneboronic acid are so similar to those described above for benzeneboronic acid that they do not require further discussion.

Finally, as for benzeneboronic acid, the 2-phenylethaneboronic acid difference map also shows a 4 Å negative depression near the Ser-221 Oγ position in the tetrahedral adduct, which we again interpret as being due to the unexpectedly small displacement of this oxygen atom from its position in the native enzyme.

A second 2-phenylethaneboronic acid molecule is bound noncovalently at a site on the enzyme surface near that which we have identified previously as the probable binding locus for the leaving group residue P1 of a polypeptide substrate (3). The orientation of the inhibitor in this secondary binding mode is also depicted in Fig. 3.

In contrast to the inhibitor molecule covalently attached to Ser-221, in the inhibitor molecule at the secondary site Ca', Ca'2, and Ca'3 lie in the plane of the benzene ring, which is rotated by 90° about the Ca'C—Ca'3 bond from a geometry that would minimize nonbonded electron repulsions. Preference for this evidently somewhat sterically strained rotomer may be due to (a) a favorable net energy gain attributable to π-π interaction with the side chain of Tyr-217, (b) a strong hydrogen bond (2.8 Å) from one boron hydroxyl to the backbone carbonyl oxygen of Asn-218, and (c) interactions with the primary, covalently bound inhibitor at the active site.

1. The phenyl group of the inhibitor in the secondary site is sandwiched between the side chains of His-64 and Tyr-217, shown in Fig. 3 as they are positioned in the native enzyme. A number of close nonbonded contacts between the inhibitor and the aromatic ring of Tyr-217 are visible. These must be at least partially responsible for slight movement of the Tyr-217 side chain which is evidenced by negative and positive density at the 4 σ level on opposite sides of the Tyr-217 aromatic ring. This density redistribution is consistent with small rotations about the Cα—Cβ and Cβ—Cγ bonds in Tyr-217 which have the effect of relieving neighboring group repulsions and also maximizing π-π interaction between the two aromatic groups. The secondary inhibitor phenyl group is also in van der Waals contact with His-64 at Ca, C82, and Ne1. In addition a close contact of 3.3 Å occurs between the aromatic ring C91 of the inhibitor and Ce of Met-222.

2. A broad region of positive difference density reaches heights of 4 σ over an extensive area coincident with the positions of C, O, Ca, and Cβ of Asn-218 in our current model of the active enzyme. The apparent absence of adjacent negative density suggests that the thermal disorder of this residue has been reduced as a consequence of hydrogen bond formation to one of the inhibitor hydroxyl groups.

3. Since the secondary, noncovalently bound molecule of 2-phenylethaneboronic acid must always encounter another inhibitor molecule in the primary binding site (because the latter is essentially 100% occupied) the possibility exists that additional stabilization energy is available through mutual interaction of the two. In fact the boronic acid hydroxyl group near Asn-218 appears to accept a strong hydrogen bond (2.5 Å) from the adjacent covalently bound inhibitor hydroxyl group situated in the oxyanion hole. Also, the remaining boronic acid hydroxyl group in the secondary binding site makes a hydrogen bond 3.0 Å long with the remaining covalent inhibitor hydroxyl group located near the imidazole side chain of His-64.

**DISCUSSION**

A number of papers have appeared recently dealing with the inhibition of serine proteases by boronic acids. The weight of evidence seems to favor nucleophilic attack by the enzyme at the inhibitor boron atom resulting in creation of a new covalent bond. However, the kinetic data on which these arguments are based are inconclusive with respect to the nature of this covalent bond.

The uncertainty is centered principally on two points: (a) the identity of the reactive nucleophile at the enzyme's active site and (b) the coordination number of the inhibitor boron atom. Antonov et al. (11) have studied the inhibition of chymotrypsin by boric acid and α-benzeneboronic acids. They postulated a tetrahedral coordination sphere for boron with a covalent link to the enzyme through Nε2 of His-57. A similar conclusion was reached by Philipp and Bender (8) from their inhibition studies of chymotrypsin and subtilisin BPN' with a number of arylboronic acids. They also suggested covalent attachment to boron by both the active site histidine and serine as another possibility. Koehler and Lienhard (5), and more recently Lindquist and Terry (7), have interpreted the kinetics of chymotrypsin and subtilisin Carlsberg inhibition by 2-phenylethaneboronic acid and benzeneboronic acid as consistent with those of the earlier investigations, but offer the additional suggestion that a bonding scheme such as that shown in Scheme I, with the boron atom covalently linked to the catalytic site serine Oγ, is also in accord with the observed kinetics.

Our results unambiguously define the geometry of these two boronic acid inhibitors at the active site of subtilisin BPN' and, by analogy, at the active site of chymotrypsin. There is clearly a covalent bond between Oγ of the catalytic Ser-221 and the inhibitor boron atom. Furthermore, the boron atom is coordinated tetrahedrally, with the two additional boronic acid oxygen atoms lying in the oxyanion hole and at the leaving group site deduced from our previous inhibitor binding studies (3). The imidazole side chain of His-64 is, at the closest approach, more than 4 Å from the boron and therefore cannot be covalently bonded to the inhibitor. The His-64 side chain does, however, play an important role in stabilizing the complex by virtue of its ability to form a hydrogen bond with the boronic acid hydroxyl group occupying the leaving group site. The detailed placement of protons within the catalytic site of the boronic acid-subtilisin adducts is uncertain since hydrogen atoms cannot be observed directly in protein crystal structures determined by x-ray diffraction. Evidence bearing on this question will be discussed elsewhere.3

More importantly, these tetrahedral boronic acid adducts are isosteric with and are stabilized by the same interactions earlier proposed for stabilization of the tetrahedral intermediate implicated in the enzymic mechanism (3). Briefly to summarize what is probably the most significant aspect of this structure, it provides a mechanism for stabilizing tetrahedral intermediates by means of two hydrogen bonds from the enzyme to the carbonyl oxygen of the substrate peptide or ester bond being hydrolyzed. In
subtilisin, the hydrogen bond donors are the backbone amido group of catalytic Ser-221 and the side chain amido group of Asn-155; in chymotrypsin or trypsin they are the backbone amido groups of the catalytic Ser-195 and of Gly-193. It is a reasonable assumption that the geometry of the transition states leading to and from the tetrahedral intermediates implicated in both acylation and deacylation must closely resemble those tetrahedral intermediates. The point to be emphasized is that these stabilizing hydrogen bonds cannot be made in either the Michaelis complex or in the acyl enzyme. Consequently, and this is the essence of the matter, the enzyme will stabilize the transition states with respect to both Michaelis complex and acyl enzyme, thereby providing the catalytic rate enhancement which is its function (12).

During the course of work described here, crystallographic studies of the interaction of trypsin with the pancreatic and soybean trypsin inhibitors have revealed the existence of a susceptible peptide linkage on the inhibitor in which the carboxyl carbon assumes an approximately tetrahedral configuration in the complex (13, 14). Hydrogen bonds are donated by the two trypsin backbone amido groups in the manner described above. In the case of the pancreatic trypsin inhibitor-trypsin complex, the reactive serine Oy has penetrated the van der Waals sphere of the carboxyl carbon donated by the susceptible inhibitor peptide bond, but, owing to constraints imposed by intermolecular interactions remote from the catalytic site, it has been frozen at some position intermediate between van der Waals contact and covalent bond formation (13). Nevertheless the carboxyl carbon is distorted toward tetrahedral geometry and the developing negative charge on the carboxyl oxygen is apparently at least partially stabilized by the hydrogen bonding scheme discussed above. The soybean trypsin inhibitor-trypsin complex is somewhat different in that a true tetrahedral configuration for the carboxyl carbon seems to give the best fit to the experimental electron density distribution (14).

We are now in a better position to understand the very rapid reversible covalent inhibition of serine proteases by boronic acids (8, 15). According to Hammond’s postulate (16) for a reaction in which the transition state has structural features intermediate between the starting materials and the products, any factors which stabilize these features in the products will also stabilize them in the transition state, relative to the starting materials. It is reasonable to assume that for covalent boronic acid adduct formation the transition state will have a geometry intermediate between planar and tetrahedral. Since the enzyme-bound product is stabilized via the hydrogen bond system described below, it is not surprising that formation and dissociation occurs rapidly, owing to a lowered free energy of activation.

To what extent does the 2-carbon atom difference between the side chains of benzeneboronic acid and 2-phenylethanesboronic acid influence their binding? In the case of benzeneboronic acid, the requirement for optimal fitting of the aromatic group into the S1 specificity groove has been moderated by the need to preserve favorable interactions with the boronic acid functional group at the catalytic site and by the absence of an intervening extender like the ethylene bridge in 2-phenylethanesboronic acid. Consequently, there is no discernible movement of the enzyme backbone segment 125-127 when benzeneboronic acid binds, in contrast to the effect of 2-phenylethanesboronic acid binding and to results obtained earlier with polypeptides and chloromethyl ketone analogs (2, 3). Furthermore, because of the shorter length of its hydrophobic group, benzeneboronic acid shows no secondary binding as it cannot simultaneously take advantage of the favorable enzyme-inhibitor interactions observed for the B(OH)2 group and for the aromatic ring in the secondary binding site occupied by 2-phenylethanesboronic acid.

The noncovalently associated molecule of 2-phenylethanesboronic acid at the secondary binding site is located in the same general region on the enzyme surface as that which functions as the binding site for N-benzoyl-L-arginine (17). Although Tyr-127 participates in favorable enzyme inhibitor interactions with both of these molecules, their exact positioning is strongly influenced by differences in their side chain polarities. Thus, the phenyl group of the boronic acid is held in a hydrophobic pocket, one side of which incorporates the side chain of Tyr-217. In contrast, the arginine side chain of benzoylarginine extends along the opposite face of the Tyr-217 aromatic ring, leaving the charged guanidinium group exposed to solvent. In passing, it should be noted also that the absence of any covalent adduct formation between Oy of Ser-221 and the boronic acid molecule at the secondary binding site argues against that site’s functioning as an alternative productive binding site in substrate hydrolysis (18).

It is now apparent that many of the most potent inhibitors of serine proteases readily attain stable tetrahedral configurations about a reactive electrophilic atomic center. In the case of diisopropyl phosphofluoridate (19, 20), phenylmethylsulfonifluoride (4) and arsionic acids (21) tetrahedral geometry pre-exists in the uncomplexed molecule as well as in the covalent adduct with the catalytic serine. For boronic acids and aldehyde substrate analogs (22), a planar-tetrahedral transition can occur as a consequence of nucleophilic attack by the active site serine and subsequent stabilization of tetrahedral geometry on the enzyme surface. We suggest that a characteristic common to all of these inhibitor geometries is that in each case the inhibitor molecule can be stabilized as an analog of the enzymatic tetrahedral intermediate in a manner similar to that described here for the boronic acid adducts.

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