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2-Phenylethaneboronic Acid, a Possible Transition-State Analog for Chymotrypsin*

Karl A. Koehler and Gustav E. Lienhard†

ABSTRACT: 2-Phenylethaneboronic acid, hydrocinnamamide, and 2-phenylethanesulfonate are competitive inhibitors of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate. The dissociation constants (K_i) of the enzyme–inhibitor complexes have been determined as a function of pH over the pH range between 5 and 10. 2-Phenylethaneboronic acid binds most tightly to the form of chymotrypsin in which the imidazole group of histidine-57 at the active site exists as the free base (K_i = 0.04 mM at pH 8), whereas the state of protonation of this imidazole does not significantly affect the binding of hydrocinnamamide (K_i = about 6 mM between pH 5 and 8).

2-Phenylethanesulfonate binds more tightly to the form of the enzyme in which the imidazole is protonated (K_i = 2.9 mM at pH 5) than it does to the form in which the imidazole is unprotonated (K_i = 30 mM at pH 8). These facts are used in conjunction with the chemistry of boronic acids and the present knowledge of the structure and mechanism of action of chymotrypsin to deduce that one possible structure for the complex between chymotrypsin and the boronic acid is the structure in which the hydroxyl group of serine-195 has added to boron to form a negatively charged tetrahedral adduct and in which the imidazole is protonated, $^{+}HN\text{–}E\text{–}CH_2\text{–}O\text{–}B(R)\cdot(OH)_2^{-}$. Such a structure is an approximate analog of the transition states for acylation and deacylation of serine-195 which occur during catalysis.

A transition-state analog for an enzyme is a compound which forms a stable complex with the enzyme in which complex the structure of the analog resembles the substrate portion of the transition state (structure of highest energy) for the enzymatic conversion of bound substrate into bound product (Pauling, 1948; Wolfenden, 1969). The crystal structure of an

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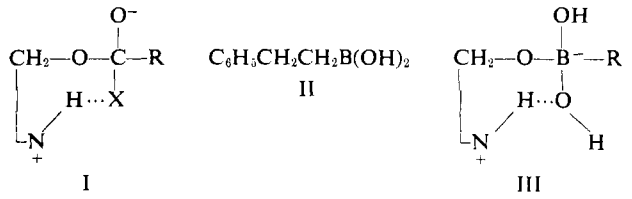
enzyme–transition-state analog complex should reveal a great deal about the mechanism of enzymatic catalysis, since it should show those interactions between enzyme and substrate which occur in the transition state and which account for catalysis by lowering the energy of the transition state. Moreover, conformational changes of the enzyme which accompany catalysis should be revealed. This paper reports preliminary efforts to find a transition-state analog for α-chymotrypsin, the crystal structure of which has been determined (Matthews *et al.*, 1967).

Chymotrypsin catalyzes acyl-transfer reactions. Catalysis proceeds by way of acylation and deacylation of the hydroxyl group of seryl residue 195 (Bender and Kézdy, 1965). Both the formation and breakdown of the acyl-enzyme intermediate are facilitated by proton transfer to and from the imidazole group of histidine-57 which is at the active site (Bender and Kézdy, 1965; Steitz *et al.*, 1969). The transition states for many nonenzymatic acyl-transfer reactions probably closely resemble in structure the high-energy tetrahedral intermediates which occur in these nonenzymatic reactions (Jencks, 1969), and it seems likely that the transition states for acylation and deacylation of chymotrypsin also resemble tetrahedral intermediates (Jencks, 1969). Such a tetrahedral intermediate for chymotrypsin-catalyzed reactions is crudely represented in I; here X is the leaving group in the case of acylation and the nucleophile in the case of deacylation.



I

$C_6H_5CH_2CH_2B(OH)_2$

II

III

The potential transition-state analog which we have investigated is 2-phenylethaneboronic acid (II). Boronic acids ionize by the addition of hydroxide ion to the trigonal boron to form negatively charged tetrahedral adducts (Lorand and Edwards, 1959; Bell *et al.*, 1967). Consequently, it seemed possible that the boronic acid might bind to chymotrypsin as the transition-state analog structure, III.[1] This structure is approximately isosteric with I, since the C–B and B–O bond lengths for tetrahedral boron are about 1.57 and 1.48 Å, respectively (Lappert, 1967; Ross and Edwards, 1967), and the C–C and C–O single-bond lengths are 1.54 and 1.43 Å, respectively (Pauling, 1960). Also, III bears the same net charge as I. The 2-phenylethyl group was used in order to satisfy a specificity requirement of chymotrypsin (Brot and Bender, 1969). The work reported herein shows that 2-phenylethaneboronic acid may be a transition-state analog for α-chymotrypsin.

Materials

α-Chymotrypsin, the three-times-crystallized activation product of three-times-crystallized zymogen, dialyzed and salt-free, was purchased from Worthington Biochemical Corp.

Methyl hippurate was purchased from Mann Research Laboratories and recrystallized twice from water, or it was prepared from hippuric acid and methanol by the method of Mill and Crimmin (1957).

Hydrocinnammamide was prepared as follows. Hydrocinnamoyl chloride (25 g), which had been purified by distillation, was dissolved in 500 ml of dry benzene. Anhydrous ammonia gas was passed into the solution until precipitation of ammonium chloride ceased. The ammonium chloride was removed by filtration and washed with benzene. The white solid remaining after evaporation of the combined filtrate and benzene washes was recrystallized four times from chloroform and petroleum ether (bp 35–40°). Several subsequent recrystallizations from water yielded white crystals of mp 97–99°, lit. mp 99–100° (Kikugawa *et al.*, 1969), which were shown by titration to be free of ammonium chloride. *Anal.* Calcd for $C_9H_{11}NO$: C, 72.48; H, 7.38. Found: C, 72.34; H, 7.44.

The sodium salt of 2-phenylethanesulfonic acid was prepared according to the method of Evans *et al.* (1927). The product was recrystallized twice from distilled water and washed twice with acetone. Titration of the product with HCl demonstrated the absence of sodium sulfite. *Anal.* Calcd for $C_8H_9NaSO_3$: C, 46.15; H, 4.36. Found: C, 45.68; H, 4.36.

The procedure of Bean and Johnson (1932) was used for the preparation of 2-phenylethaneboronic acid (see Yabroff *et al.*, 1934). The compound was recrystallized from water, allowed to dry in the air for several days, and then stored under argon in order to prevent its oxidation. *Anal.* Calcd for $C_8H_{11}BO_2$: C, 64.06; H, 7.39. Found: C, 63.71, 63.62; H, 7.30, 7.34. The compound gave the following proton magnetic resonance spectrum in deuterated dimethyl sulfoxide, the signals of which are expressed as δ (apparent multiplicity, relative integrated intensity of the signal, assignment to hydrogen atoms in the compound), where δ is the chemical shift of the signal in parts per million relative to the external standard of tetramethylsilane in chloroform: 0.85 (triplet with J = 7–8 Hz, 2 H, CH_2–B), 2.60 (triplet with J = 7–8 Hz, 2 H, phenyl–CH_2), 7.18 (singlet, 5 H, phenyl hydrogens), and 7.50 (singlet, 2 H, B–$(OH)_2$). Titration of 0.02 M 2-phenylethaneboronic acid under nitrogen with 0.1 N NaOH at 22–24° gave a pK_a value of 10.0 (lit. pK_a value at 25°, 10.0 (Yabroff *et al.*, 1934)) and an equivalent weight of 151 (mol wt 150). A titration was also carried out in the presence of 0.5 M mannitol (Branch *et al.*, 1934); the apparent pK_a value was 7.1 and the equivalent weight was 150.

Methods

The initial rates of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate were measured by the consumption of sodium hydroxide in a Radiometer pH-Stat apparatus. All rates were determined at 25.0° under argon with 15-ml reaction mixtures that contained 0.1 M KCl and 10^{-3} M potassium phosphate. Each kinetic run was organized as follows. A solution of buffer, substrate, and, where present, inhibitor was incubated in the thermostatted reaction vessel at 25° for about 10 min; the reaction was initiated by the addition of a small aliquot of chymotrypsin in 10^{-3} N HCl, and the uptake of NaOH with time was recorded.

The concentration of the enzyme was always such that less than 10% of the substrate was hydrolyzed during the initial several minutes of reaction, and the consumption of base during each kinetic run was linear with respect to time for at least this period. Above pH 8 the rate of hydroxide ion catalyzed hydrolysis of methyl hippurate contributed significantly to the observed initial rate of base uptake. The rates of the non-

[1] Since the transition state and any metastable tetrahedral intermediate from which it may be formed are probably similar in energy and structure (Hammond, 1955), III can also be considered to be a metastable intermediate analog structure.

enzymatic hydrolysis were measured by the pH-Stat method under conditions identical with the above ones, and each initial rate for the enzymatic reaction was obtained by subtracting the initial rate of base uptake in the absence of enzyme from that in its presence. In the most unfavorable case, which was one with a high concentration of methyl hippurate at pH 10, this background rate accounted for 74% of the overall rate. Other control experiments showed that in the absence of methyl hippurate there was no base uptake with enzyme and 2-phenylethaneboronic acid, hydrocinnamamide, or 2-phenylethanesulfonate over the time period normally used for measurements.

Stock solutions of chymotrypsin were prepared in 10^{-3} N HCl. These were assayed for active enzyme before and after each series of rate measurements by measuring the rate of hydrolysis of 8 mM methyl hippurate at pH 7.28. The activity of the enzyme in the stock solutions, which were stored at 2°, did not change over a period of 1 month. In this standard assay a rate of 9×10^{-6} M min^{-1} corresponded to an enzyme concentration of about 10^{-6} M, based upon mol wt 24,800 and the weighed amount. This rate constant of about 9 min^{-1} under these conditions is similar to values which have been reported previously (Bernhard *et al.*, 1960; Cuppett and Canady, 1970). The concentrations of chymotrypsin which were used ranged from 3×10^{-6} to 3×10^{-5} M. At these concentrations and at 0.1 M ionic strength, significant polymerization does not occur with the enzyme alone in the pH range from 5 to 8 (Shiao and Sturtevant, 1969; Steiner, 1953). We have found that the rate of hydrolysis of 20 mM methyl hippurate at pH 5 and pH 7.3 and of 0.9 and 9 mM methylhippurate at pH 10 is directly proportional to the concentration of chymotrypsin over the range from 3×10^{-6} to 3×10^{-5} M. These findings suggest that there is no significant polymerization in this concentration range at pH 10 as well as at pH values from 5 to 8 (Inagami and Sturtevant, 1965).

At each pH value with each inhibitor, sets of initial rate measurements were made at four or five substrate concentrations in the presence of no inhibitor and in the presence of one or more constant concentrations of inhibitor. For sets below pH 9.0, the substrate concentrations varied from a value of about one-third the K_m to a value of about three times the K_m. Above pH 8.5 the K_m value for methyl hippurate increases; and at pH values from 9.0 to 10.0, the highest substrate concentration which was used was only equal or less than the K_m.

Results

Figure 1 presents kinetic data for the inhibition of the chymotrypsin-catalyzed hydrolysis of methyl hippurate by 2-phenylethaneboronic acid at pH 7.28. The fact that the $1/v$ *vs.* 1/[S] plots are linear and all intersect at the $1/v$ axis demonstrates that the inhibition is competitive and can be described by the equation for competitive inhibition (Dixon and Webb, 1964)

$$\frac{1}{v} = \frac{K_m(1 + [I]/K_i)}{k_{cat}[E]_t[S]} + \frac{1}{k_{cat}[E]_t} \quad (1)$$

where $[E]_t$, [S], and [I] are the concentrations of enzyme in all forms, substrate, and inhibitor, respectively. Similarly, we found that the inhibition by 2-phenylethaneboronic acid at other pH values between 5 and 10 is competitive and that the inhibitions by hydrocinnamamide and 2-phenylethanesulfonate over the pH range from 5 to 10 are competitive. The values of K_i, which is the dissociation constant of enzyme inhibitor complex, were derived from the $1/v$ *vs.* 1/[S] plots by use of the equation

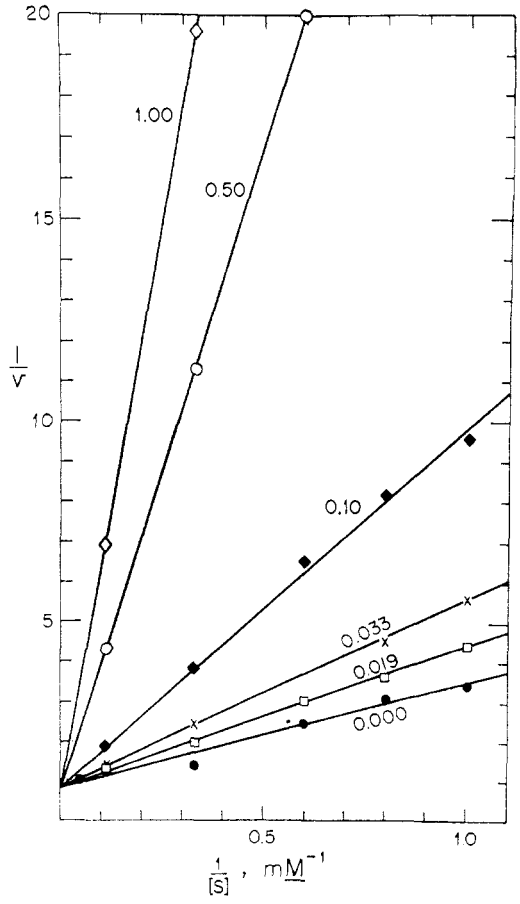


FIGURE 1: Inhibition of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate by 2-phenylethaneboronic acid at pH 7.28. The reciprocal of the initial velocity (v) which has been normalized to one concentration of enzyme and is expressed in arbitrary units of base consumption per minute is plotted against the reciprocal of the substrate concentration ([S]). The number on each plot is the millimolar concentration of inhibitor at which the set of rates were measured.

$$K_i = \frac{[I]\text{slope}_u}{\text{slope}_i - \text{slope}_u} \quad (2)$$

in which slope_u is the slope of the plot for the set without inhibitor ($K_m/k_{cat}[E]_t$) and slope_i is the slope of the plot for each set with inhibitor present, ($K_m(1 + [I]/K_i)/k_{cat}[E]_t$), with all rates normalized to the same amount of active enzyme.

The values of K_i are plotted as a function of pH in Figure 2. These profiles can be adequately explained in terms of the dissociation of two groups of the enzyme according to eq 3 in which K_{e1}, K_{e2}, K_{ei1}, and K_{ei2} are acid dissociation constants and K_{1i}, K_{2i}, and K_{3i} are the dissociation constants of the

$$\begin{array}{ccc} EH_2 + I & \overset{K_{1i}}{\rightleftharpoons} & EH_2I \\ K_{e1}\downarrow\uparrow & & \downarrow\uparrow K_{ei1} \\ EH + I & \overset{K_{2i}}{\rightleftharpoons} & EHI \\ K_{e2}\downarrow\uparrow & & \downarrow\uparrow K_{ei2} \\ E + I & \overset{K_{3i}}{\rightleftharpoons} & EI \end{array} \quad (3)$$

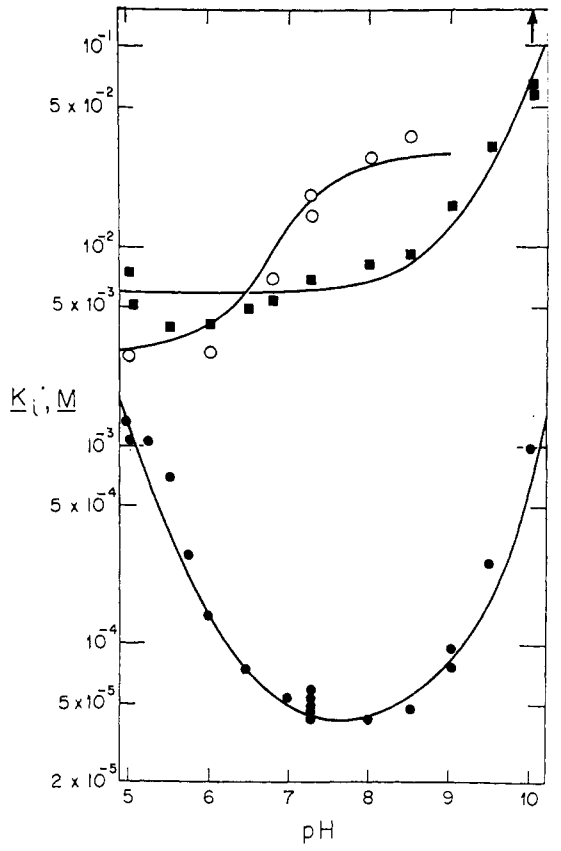


FIGURE 2: Semilogarithmic plots of K_i *vs.* pH, for the inhibition of α-chymotrypsin-catalyzed hydrolysis of methyl hippurate by 2-phenylethaneboronic acid (●), hydrocinnamamide (■), and 2-phenylethanesulfonate (○). The solid lines have been calculated from the derived parameters (see the text). The value for 2-phenylethanesulfonate at pH 10 (↑) is a lower limit based upon the fact that no significant inhibition was detected with 80 mM compound.

various enzyme–inhibitor complexes. Table I summarizes the values for these various constants for each of the three inhibitors. The solid lines in Figure 2 have been calculated using these constants and eq 4, which defines the relationship be-

$$K_i = K_{2i}\left(\frac{(H^+)/K_{e1} + 1 + K_{e2}/(H^+)}{(H^+)/K_{ei1} + 1 + K_{ei2}/(H^+)}\right) \quad (4)$$

tween the value of K_i and the constants. This analysis does not include a consideration of the ionization of 2-phenylethaneboronic acid (pK_a = 10.0). At pH values above 9 a significant percentage (25% at pH 9.5, 50% at pH 10.0) of the total concentration is the boronate anion. From our data it is not possible to decide whether the boronic acid anion as well as the acid itself is an inhibitor of chymotrypsin. However, it is certain that this partial ionization of the acid cannot account for the 25-fold increase in the value of K_i between pH 8 and 10; the increase must be due largely to the dissociation of a group on the enzyme.

The rate measurements also yielded accurate values of K_m, the Michaelis constant, and k_{cat}, the turnover number, for methyl hippurate as a function of pH between pH 5 and 9. Cuppett and Canady (1970) previously have described the dependence of K_m and k_{cat} on pH over the pH range from 6 to 10 under conditions which are the same as the ones used here. Our values are equal to their values ±20% between pH 6 and 9.

Discussion

For the following reasons, we conclude that the three inhibitors, 2-phenylethaneboronic acid, hydrocinnamamide, and 2-phenylethanesulfonate, bind at the active site of chymotrypsin. (1) Each inhibitor contains the $C_6H_5CH_2$ group, which is a specificity determinant of chymotrypsin that can be bound in a hydrophobic pocket at the active site (Steitz *et al.*, 1969). (2) The inhibitions are competitive with respect to the substrate. (3) The strengths of the interactions of chymotrypsin with the boronic acid and the sulfonate depend on the state of ionization of two groups on the enzyme with pK values of 6.4 and 8.9; in the case of the amide only the group with the pK value of 8.9 is evident (Table I). The group with pK of 6.4 can be identified as the imidazole of histidine-57. This imidazole is known to be at the active site (Steitz *et al.*, 1969), and kinetic investigations have shown that its pK in the free enzyme is about 6.5 (Himoe *et al.*, 1967). Ionization of the group with a pK of 8.9 results in a decreased affinity of the enzyme for the inhibitors (Figure 2). This behavior identifies the group as the α-amino group of isoleucine-16. Hess *et al.* (1970) have shown in several ways that the pK of this group is about 8.7 and that deprotonation of it is accompanied by a conformational change of the enzyme to a form which binds substrates and substrate analogs less tightly.

In addition to the hydrophobic pocket and the imidazole group of histidine-57, the active site of chymotrypsin contains the hydroxyl group of serine-195, which undergoes acylation and deacylation in the course of catalysis, and the β-carboxylate group of aspartate-102. In that form of chymotrypsin which has the imidazole of histidine-57 protonated this carboxylate group is hydrogen bonded to that NH of this imidazolium group which is furthest from the serine hydroxyl (Blow *et al.*, 1969). Its location in the form of the enzyme in which the imidazole exists as the free base has not been unambiguously determined but is probably the same as its location in the form with protonated imidazole (Blow *et al.*, 1969). On the basis of this picture of the active site and the pH dependence of K_i, there appear to be eight possible structures for the complex which occurs between 2-phenylethaneboronic acid and chymotrypsin in the pH range between 5 and 9. These are presented in Figure 3. Note that the net charge of each structure is −1. This net charge is required because of the pH dependence of K_i, which shows that the binding of 2-phenylethaneboronic acid to the form of the enzyme in which the imidazole of histidine-57 is protonated is less than one-fortieth as strong as its binding to the form in which the imidazole is unprotonated (Figure 2 and Table I). We will consider the merit of each of these structures.

A1. In structure A1 the boronic acid simply binds noncovalently to chymotrypsin. The following argument leads to the conclusion that this structure is not the real one. The C–B-$(OH)_2$ group has a planar structure with the C–B and B–O bond lengths of about 1.57 and 1.37 Å, respectively, and bond angles of about 120° (Lappert, 1967; Ross and Edwards, 1967; Bell *et al.*, 1967); it is, within 0.13 Å, isosteric with an amide group (Pauling, 1960). Moreover, the hydrogen-bonding interactions of the boronic acid and amide groups can be similar, because OH can be either a hydrogen-bond donor or acceptor. Finally, hydrocinnamamide appears to bind noncovalently to chymotrypsin, since its K_i value of 8 mM at pH 7.9 is very close to the K_i value of 4 mM which has been reported for competitive inhibition by 2-phenylethanol under the same conditions (Wallace *et al.*, 1963). For these reasons, if structure A1 were correct, the interaction between chymotrypsin

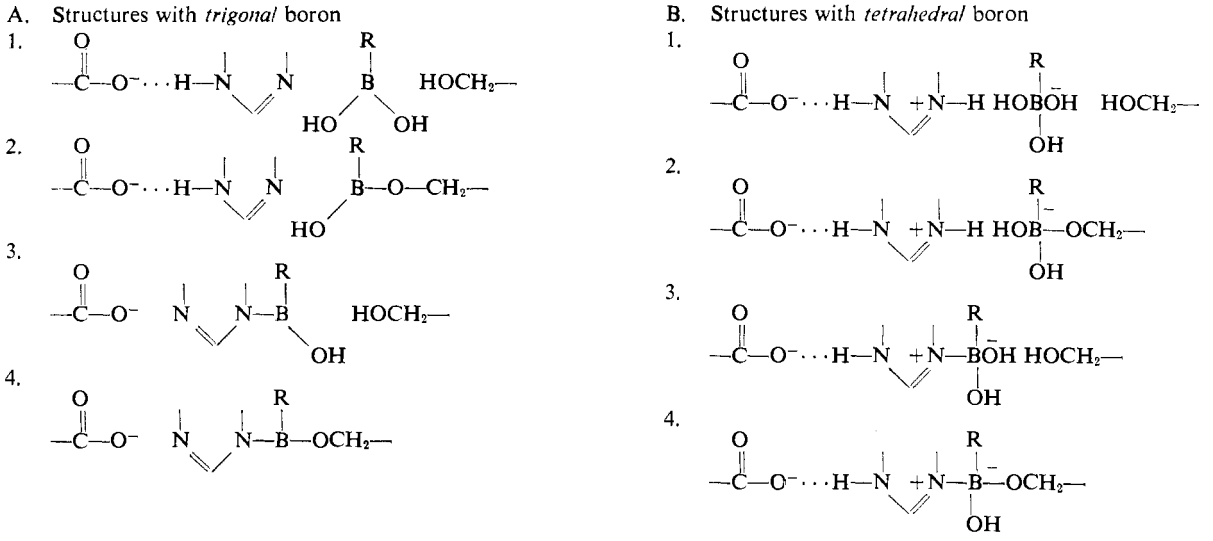


FIGURE 3: Schematic representations of the possible structures of the complex between chymotrypsin and 2-phenylethaneboronic acid. The groups are: CO_2^- of aspartate-102, imidazole of histidine-57, and CH_2OH of serine-195.

TABLE I: Constants for the Inhibition of Chymotrypsin by 2-Phenylethaneboronic Acid, Hydrocinnamamide, and 2-Phenylethanesulfonate.[a]

Inhibitor	K_{1i} (mM)	K_{2i} (mM)	K_{3i} (mM)	pK_{e1}	pK_{e2}	pK_{ei1}	pK_{ei2}
1. 2-Phenylethaneboronic acid	>1.5	0.04	>1.0	6.4	8.8	<4.8	>10.3
2. Hydrocinnamamide	6	6	>50	*b*	9.0	*b*	>9.9
3. 2-Phenylethanesulfonate	2.9	30		6.4		7.4	

[a] See eq 3 in the text for the definition of the constants. Values of K_{1i}, K_{2i}, and K_{3i} were given by the pH-independent portions of the plots of K_i *vs.* pH in Figure 2. Values of pK_{e2} and pK_{ei2} were obtained from the data above pH 7.5 in Figure 2 by use of the relationships, $K_{3i}/K_{2i} = K_{e2}/K_{ei2}$ and $K_i = K_{2i}((H^+) + K_{e2})/(H^+)$. Values of p$K_{e1}$ and pK_{ei1} were determined from the data below pH 7.5 in Figure 2 as follows: for the boronic acid, by use of the relationships, $K_{2i}/K_{1i} = K_{e1}/K_{ei1}$ and $K_i = K_{2i}((H^+) + K_{e1})/K_{e1}$; for the sulfonate, by use of the equations $K_{2i}/K_{1i} = K_{e1}/K_{ei1}$ and $K_{e1} = K_{2i}(H^+)(K_i/K_{1i} - 1)(1/(K_{2i} - K_i))$. [b] Indeterminate, since K_i is approximately pH-independent between pH 5 and 7.5.

and 2-phenylethaneboronic acid would be similar to that between chymotrypsin and hydrocinnamamide. Since the boronic acid binds 200 times more tightly than the amide at pH 8 and since the pH dependences of K_i for the two differ markedly (Figure 2), structure A1 seems very unlikely.

A2. This structure is the boron analog of the acyl-enzyme. It is very unlikely that there is any kinetic barrier to the formation of an equilibrium amount of this structure within a few seconds, since the nonenzymatic formation and hydrolysis of unhindered boronic acid esters should occur very rapidly (Bell *et al.*, 1967) and also the enzyme may catalyze the reaction. However, the apparent pK of the active-site imidazolium group in the acyl-enzymes which are formed from both specific and nonspecific substrates is about 7 (Bender *et al.*, 1964; Hess *et al.*, 1970), whereas the pK of the imidazolium group in the complex with 2-phenylethaneboronic acid is less than 4.8 (Table I). Consequently, A2 is probably not the correct structure.

A3 and A4. These are planar structures in which the imidazole nitrogen is covalently bonded to the boron. These structures seem unlikely because their formation requires the ionization of the imidazole hydrogen atom which is hydrogen bonded to the carboxylate anion of Asp-102 and consequently the disruption of the electronic network through which this buried charge is stabilized (Blow *et al.*, 1969). Also, examination of the molecular model of α-chymotrypsin (kindly provided by Dr. W. N. Lipscomb, built according to the coordinates of Birktoft *et al.* (1969)) reveals that the phenyl group cannot fit as deeply within the hydrophobic pocket when the boron is linked to the imidazole nitrogen.

Because of the tendency of trigonal boron to form tetrahedral adducts (Bell *et al.*, 1967), structures B1–4, which contain tetrahedral, negatively charged boron, must be considered as possible structures.

B1. In this structure the boronate anion is bound noncovalently to the protonated active site (eq 5). The dissociation

$$EH_2 + I^- \rightleftharpoons EH_2 \cdots I^- \qquad (5)$$

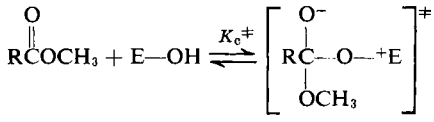
constant, K_{2i}', for the reaction in eq 5 is equal to $K_{2i}K_{HB}/K_{e1}$, where K_{HB} is the acid ionization constant of 2-phenylethaneboronic acid. The value of K_{2i}' is thus 10^{-5} mM (see Table I). In order to determine whether such a small dissociation constant would be expected for this type of interaction, we determined tne values of K_i for 2-phenylethanesulfonate. 2-Phenylethanesulfonate resembles the boronate anion in the sense

that the C–SO_3^- group is tetrahedral and negatively charged; it is not, however, exactly isosteric since the C–S and S–O bond lengths are 1.85 and 1.47 Å, respectively (Brandon and Brown, 1967), whereas the C–B and B–O bond lengths for tetrahedral boron are about 1.57 and 1.48 Å, respectively (Lappert, 1967; Ross and Edwards, 1967). Since at pH 5 carboxylate anions bind noncovalently to chymotrypsin (Steitz *et al.*, 1969) and since at pH 5 the sulfonate anion is more stable relative to its ester or conjugate acid (pK of methanesulfonic acid = −0.6) than a carboxylate anion is stable relative to its ester or conjugate acid (pK of acetic acid = 4.7), it seems almost certain that 2-phenylethanesulfonate binds to chymotrypsin in a noncovalent way. The value of the equilibrium constant for the dissociation of the complex between the sulfonate and the EH_2 form of chymotrypsin is 2.9 mM (Table I), which is 3×10^5 times greater than K_{2i}' for the boronic acid. Consequently, structure B1 seems unlikely.

It should be noted that the 2-phenylethanesulfonate binds about ten times more tightly to the active site of chymotrypsin with a net charge of zero than to the active site with a net charge of −1 (Figure 2 and Table I). A similar effect of electrostatic repulsion has been observed in the binding of carboxylate anions to α-chymotrypsin (Johnson and Knowles, 1966).

B2,3,4. On the basis of the foregoing discussion, one of these three structures is probably the most likely structure of the complex between chymotrypsin and 2-phenylethaneboronic acid. At present there is not enough information to determine the relative stabilities of these three structures. Examination of the molecular model of chymotrypsin (see A3,4) does show that where the boron is bonded to the imidazole nitrogen (B3 and 4), the phenyl group cannot fit deeply in the hydrophobic pocket.

Structure B2 is the structure analogous to the transition states of chymotrypsin-catalyzed reactions. An approximate calculation of how strongly 2-phenylethaneboronic acid would bind to chymotrypsin if its complex resembles the transition states can be made in the following way. The equilibrium constant for formation of the transition state for acylation of chymotrypsin by unbound methyl hydrocinnamate ($K_c^{\ddagger}$, eq 6) can be calculated from the rate constant for this acylation reaction, which is equal to k_{cat}/K_m (Brandt *et al.*, 1967), by use of transition-state theory.

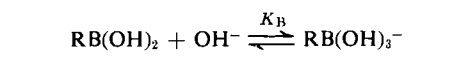


(6)

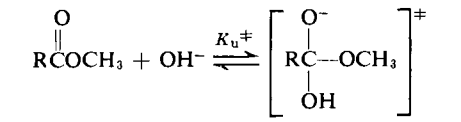
According to transition-state theory

$$K_c^{\ddagger} = \left(\frac{h}{kT}\right) k_{cat}/K_m \qquad (7)$$

where h is Planck's constant, k is Boltzmann's constant, and T is the absolute temperature (Laidler, 1969). The value of k_{cat}/K_m for the hydrolysis of methyl hydrocinnamate in water at 25°, pH 7.1, and 0.1 M ionic strength is 110 sec^{-1} M^{-1} (Canady and Laidler, 1958) so that $K_c^{\ddagger} = 110\, h/kT$ M^{-1}. The boronic acid should bind more tightly because its tetrahedral adduct is more stable, relative to the trigonal form, than is the tetrahedral-like transition state relative to the ester. A quantitative estimate of how much more tightly can be made by comparing the equilibrium constant for the addition of hydroxide ion to 2-phenylethaneboronic acid to form the tetrahedral boronate anion (K_B, eq 8) with the equilibrium constant for the addition of hydroxide ion to methyl hydrocinnamate to form the tetrahedral-like transition state of alkaline hydrolysis ($K_u^{\ddagger}$, eq 9). Since K_B is equal to the acid



(8)



(9)

dissociation constant of 2-phenylethaneboronic acid divided by the ion product of water, its value is 10^4 M^{-1}. $K_u^{\ddagger} = hk_{OH^-}/kT$ (see eq 7), where k_{OH^-} is the second-order rate constant for the alkaline hydrolysis of methyl hydrocinnamate and has a value of about 0.1 M^{-1} sec^{-1} in water at 25° (Nat. Bur. Std., 1951, 1956). Thus, the tendency of hydroxide ion to add to the boronic acid is ($K_B/K_u^{\ddagger}$) or ($10^4/0.1$)(kT/h) times greater than its tendency to add to the ester function, and the estimated dissociation constant for the transition-state analog complex B2 is $(1/K_c^{\ddagger})(K_u^{\ddagger}/K_B)$ or $(1/110)(0.1/10^4) = 9 \times 10^{-6}$ mM.

The difference between this calculated value of 9×10^{-5} mM for the dissociation constant of the complex with 2-phenylethaneboronic acid and the measured value of 0.04 mM may simply be due to the fact that although the boron adducts, B2 and 2-phenylethaneboronate anion, resemble the transition states for the reactions of methyl hydrocinnamate with chymotrypsin (eq 6) and hydroxide ion (eq 9), respectively, they are, of course, not identical in structure. The estimate has been made in such a way that the approximations in the transition-state theory (Laidler, 1969) probably cancel out. The important conclusion is that this treatment does predict, in agreement with the Results (Table I), that 2-phenylethaneboronic acid should be a potent inhibitor of chymotrypsin.

Antonov *et al.* (1970) have recently described the inhibition of chymotrypsin by boric acid and *n*-alkaneboronic acids. The pH dependences of the K_i values which they have determined are similar to the one reported herein for 2-phenylethaneboronic acid, and on this basis they postulated that the enzyme–inhibitor complexes have the B3 structure. Also, Torssell (1957) reported that cholinesterase is 50% inhibited by 4×10^{-3} mM benzeneboronic acid. Finally, it is interesting to note that Bernhard and Orgel suggested in 1959 that in the case of the inhibition of proteolytic enzymes by organic phosphate esters "it is the unstable transition state of the enzyme-substrate complex which is imitated by the inhibitor in one of its stable combinations with the enzyme."

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