The Binding of Boronic Acids to Chymotrypsin†

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ABSTRACT: The binding of 2-phenylethaneboronic acid, *trans*-β-styreneboronic acid, hydrocinnamaldehyde, and *trans*-cinnamaldehyde to α-chymotrypsin has been investigated. 2-[1,2-^{3}H]Phenylethaneboronic acid was prepared. Equilibrium dialysis with the radioactively labeled compound revealed that chymotrypsin has one binding site and that the dissociation constant is 0.05 mM at 25° and pH 7.4. The dissociation constants for styreneboronic acid, hydrocinnamaldehyde, and cinnamaldehyde at 25° and pH 7.4–7.8, determined by inhibition of the hydrolysis of methyl hippurate, are 0.026, 0.38, and 2.6 mM, respectively. The results of difference spectroscopy with styreneboronic acid show that there is one binding site for this compound and confirm the kinetically determined dissociation constant. An analysis of our results and those of others supports the hypothesis that the structures of the boronic acid–chymotrypsin complexes resemble the transition states for reaction of the corresponding substrates.

In a previous report from this laboratory, the potent inhibition of chymotrypsin by phenylethaneboronic acid (PEBA,[1] $PhCH_2CH_2B(OH)_2$) was described (Koehler and Lienhard, 1971). It was suggested that the inhibition of chymotrypsin by PEBA and other boronic acids that resemble specific substrates may be due to the formation of a complex between the boronic acid and the active site of chymotrypsin, the structure of which complex resembles the transition states for reactions catalyzed by the enzyme. In order to examine this hypothesis further, we have determined the number of binding sites for PEBA on chymotrypsin, and we have investigated the binding of *trans*-β-styreneboronic acid (SBA, $PhCH{=}CHB(OH)_2$) to chymotrypsin by ultraviolet difference spectroscopy and by a kinetic method. Aldehydes also may form transition-state-like complexes with chymotrypsin. For the sake of comparison with the boronic acids, we have determined the dissociation constants of complexes of chymotrypsin with hydrocinnamaldehyde and *trans*-cinnamaldehyde.

Materials and Methods

Three times crystallized bovine α-chymotrypsin and its diisopropylphosphoryl derivative (iPr_2P-chymotrypsin), in the form of dialyzed, salt-free, lyophilized powders, were purchased from Worthington Biochemical Corporation. The concentration of active chymotrypsin was determined by assay with *N*-*trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961). Commercial preparations of hydrocinnamaldehyde and *trans*-cinnamaldehyde were purified by fractional distillation.

SBA. The synthesis of SBA was based upon the procedure of Matteson and Bowie for the synthesis of dibutyl α-styreneboronate (Matteson and Bowie, 1965). β-Bromostyrene, obtained from K & K Laboratories, was distilled under vacuum (bp 54–55° (10 mm)), and the distillate identified as the trans isomer by its proton magnetic resonance (pmr) spectrum (Seyferth *et al.*, 1964). The trans Grignard reagent was prepared by the dropwise addition of 27 ml of β-bromostyrene to 5 g of magnesium in 50 ml of freshly dried tetrahydrofuran, under nitrogen (Yoshino *et al.*, 1964). The solution of *trans*-β-styrylmagnesium bromide was added dropwise over the period of 1 hr to a solution of 25 ml of trimethyl borate in 50 ml of anhydrous ether at −65°. The reaction mixture was allowed to warm to room temperature overnight and was then acidified with 75 ml of 2 N H_2SO_4. The aqueous phase was extracted several times with ether. The ethereal solutions were combined and extracted with 75 ml of 2 N KOH. Upon acidification of the basic extract with HCl, 3.1 g of crude product precipitated. It was recrystallized several times from water.

The proton magnetic resonance spectrum of 0.5 M SBA in deuterated dimethyl sulfoxide, expressed as signal in parts per million downfield from tetramethylsilane (relative integrated intensity of the signal, multiplicity of the signal, coupling constant, and assignment of the signal), was as follows: δ 6.12 (1 H, d, J = 18 Hz, C*H*–B) and 7.15–7.50 (6 H, complex m, PhCH). Pasto *et al.* (1969) reported similar values for these hydrogen atoms in the ethylene ester of SBA. The spectrum of SBA also showed a singlet at δ 7.85 and an enhancement of the singlet at 3.46, which is present in the spectrum of the solvent alone and is due to adventitious H_2O. The integrated intensity of the signal at δ 7.85 was one hydrogen, and the increase in the integrated intensity of the signal at 3.46 was about equivalent to one hydrogen. Since boric acid is known to undergo a polymerization by the reaction, $2HO\text{–}B< \rightarrow >B\text{–}O\text{–}B< + H_2O$, under dehydrating conditions (Ross and Edwards, 1967), the likely explanation of this part of the pmr spectrum is that 0.5 M SBA is largely dimerized in dimethyl sulfoxide, with the loss of one molecule of water per two molecules of SBA. The singlet at δ 7.85 is then due to the H of the one OH remaining bonded to the boron atom.

Titration of SBA, 10.0 mM by weight, in 0.62 M mannitol, which selectively complexes with the basic species and so lowers the pH at which conversion to the basic species is complete (Branch *et al.*, 1934), gave an equivalent weight of 148 (mol wt 148). A plot of the titration data according to the equation, $pH = pK_a + \log [base]/[acid]$, was linear with the predicted slope of 1.0. The value of the apparent pK_a in the presence of the 0.62 M mannitol is 6.60. The pK_a of the acid in the absence of mannitol was calculated from the pH values of 20 mM solutions to which 0.25, 0.50, and 0.75 equiv of NaOH were added and is 9.63 at 25°. The ultraviolet spectrum of the acid form of SBA, obtained in 44 mM KCl–20 mM potassium phosphate

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[1] Abbreviations used are: PEBA, 2-phenylethaneboronic acid; SBA, *trans*-β-styreneboronic acid; iPr_2P-chymotrypsin, chymotrypsin in which Ser-195 has been phosphorylated by reaction with diisopropyl fluorophosphate.

(pH 7.5), has λ_{max} 260 nm (ϵ 18,400 M^{-1} cm^{-1}); that of the basic form, obtained in 0.10 N NaOH, has λ_{max} 254 (ϵ 16,800 M^{-1} cm^{-1}).

Aliphatic boronic acids, in solid form, have been found to be susceptible to slow oxidation by molecular oxygen, whereas in the presence of water the acids are stable (Snyder *et al.*, 1938). SBA also appears to behave in this manner. The data from the titration, in the presence of mannitol, of a solution of SBA prepared from solid compound that had been recrystallized some days previously and left under air gave a nonlinear plot of pH *vs.* log [base]/[acid]. Also, the molar absorbancy determined with this solution was lower than the value given above. The results reported above and throughout this paper were obtained with a compound that was recrystallized from water, allowed to dry overnight in ai, and then dissolved in water. The titration behavior and ultraviolet spectra of such solutions did not change over a period of several weeks.

[1,2-^3H]PEBA. In a preliminary experiment, 74 mg of SBA in 15 ml of ethyl acetate was reduced with H_2 at atmospheric pressure and 50 mg of 10% palladium on charcoal (Pasto *et al.*, 1969). The product was identified as PEBA by its pmr spectrum (Koehler and Lienhard, 1971). This reduction was then performed for us with $^3H_2$ by the New England Nuclear Corporation. The tritiated compound was diluted with unlabeled PEBA (Koehler and Lienhard, 1971) and recrystallized from water to constant specific activity. The specific activities, in counts per minute per micromole, after each recrystallization were as follows: first, 9.60×10^5; second, 8.15×10^5; third, 7.50×10^5; fourth, 7.65×10^5. Radioactivity was measured by the use of a Packard Tri-Carb liquid scintillation spectrometer, with Bray's solution as the scintillation fluid (Bray, 1960). Since solid PEBA also undergoes slow oxidation by molecular oxygen, the labeled compound was stored as an aqueous solution, prepared from the finally recrystallized compound after it had dried overnight in air.

Equilibrium Dialysis. Commercial cellulose dialysis tubing was washed once with boiling 5 mM NaOH, then five times with boiling 20 mM $NaHCO_3$–0.5 mM disodium ethylenediaminetetraacetic acid, and finally three times with boiling glass-distilled water. Dialysis was carried out in cells obtained from TechniLab Corporation that have a capacity of 1.0 ml per chamber. In each experiment, 0.50 ml of a mixture of tritiated PEBA, protein, and buffer was dialyzed against 0.50 ml of buffer. During dialysis the cell was gently shaken in a water bath at 25.0°. After 6 and 9 hr of dialysis, aliquots were removed from each chamber for determinations of the radioactivity, from which the total concentration of PEBA in each chamber was calculated. Equilibration was complete within 6 hr. Preliminary experiments showed that the half-time for equilibration of the boronic acid alone was 35 min and that less than 2% of the boronic acid was bound to the membrane. In order to determine whether chymotrypsin and iPr_2P-chymotrypsin passed through the membrane, we measured the absorbance at 280 nm of solutions from both sides of the membrane, after the equilibration period. In each case less than 6% of the ultraviolet-absorbing material passed through the membrane. Under the conditions used for dialysis (see the legend of Figure 1), the activity of chymotrypsin in the absence of PEBA decreases by 15% during the 6-hr period, probably because of autolysis. Since 0.2 mM PEBA reduced the per cent inactivation to less than 3%, the initial concentration of active chymotrypsin was used in the calculation of the concentration of uncomplexed enzyme.

Kinetic Measurements. The pH-Stat method that was used to measure the initial rates of chymotrypsin-catalyzed hydrolysis of methyl hippurate has been described in detail in an earlier paper (Koehler and Lienhard, 1969). Each reaction mixture contained 10^{-5} M potassium phosphate–0.10 M KCl.

Difference Spectra. Difference spectra were measured with matched, two-compartment cuvets (path length, 0.44 cm per compartment) and a Cary Model 17 spectrophotometer. The reaction mixtures were prepared from stock solutions by pipetting with Lang-Levy type λ pipets. In order to minimize error, we used the same pipets to prepare the sample and reference mixtures. In experiments with chymotrypsin, the chymotrypsin was added to the rest of the reaction mixture within 15 min of recording the spectrum, from a stock in 1 mM HCl. The mixtures were prepared and the spectra were taken at ambient temperature, which was 24–25°.

The maximal absorbance *vs.* air of the mixtures used for difference spectroscopy in the 240–300-nm range varied from 0.4 to 2.4. In order to test whether the spectrophotometer gives the same difference spectrum regardless of the total absorbance, we obtained spectra of the following solutions: 0.050 mM SBA in H_2O (compartment 1)–1.00 mM tryptophan (compartment 2) *vs.* 0.050 mM SBA in 10 mM NaOH (compartment 1)–1.00 mM tryptophan (compartment 2); 0.050 mM SBA in H_2O (compartment 1)–H_2O (compartment 2) *vs.* 0.050 mM SBA in 10 mM NaOH (compartment 1)–H_2O (compartment 2). This concentration of tryptophan has an absorbance of 2.5 at 280 nm. The two spectra were the same except that the peak in the spectrum with tryptophan present was broader on its short wavelength side by about 1 nm.

Difference spectra were always recorded with the spectrophotometer operating in the automatic slit mode. The slit control setting was adjusted so that the maximum slit width was 0.7 mm or less. The difference spectrum of the acidic *vs.* basic form of SBA in the presence of 1 mM tryptophan described above was unchanged over a range of slit control settings that gave maximum slit widths ranging from 0.25 to 1.9 mm.

Chymotrypsin is known to aggregate at higher concentrations in aqueous solution. At 20°, pH 7.8, and ionic strength 0.28 M, which are conditions similar to the ones that we have used here for the equilibrium dialysis and difference spectroscopy, the equilibrium constant for dimerization is about 2000 M^{-1} (Nichol *et al.*, 1972). Using this value, we reckon that at the highest concentration of chymotrypsin used (0.083 mM), 20% of the chymotrypsin is dimerized in the absence of the boronic acids. In our subsequent calculations, we have not taken dimerization into account, since the percentage is low and since at pH 7.8 dimeric chymotrypsin binds $PhC{\equiv}CCO_2^-$ almost as tightly as monomeric chymotrypsin does (Nichol *et al.*, 1972).

Results

Binding of PEBA to Chymotrypsin. The binding of PEBA to chymotrypsin was measured by equilibrium dialysis with radioactively labeled compound. Figure 1 presents the data in the form of a Scatchard plot, which is based upon the equation for binding to *n* independent and identical sites per molecule (eq 1)

$$\frac{[\text{bound PEBA}]}{[\text{free PEBA}][E]_t} = \frac{n}{K_d} - \frac{[\text{bound PEBA}]}{K_d[E]_t} \quad (1)$$

(Gutfreund, 1972). In this equation, $[E]_t$ is the total concentration of active enzyme and K_d is the intrinsic dissociation constant of the complex. Within experimental error, the data obey eq 1. There is one site per molecule of chymotrypsin, and the value of the dissociation constant is 0.05 mM. Previously, it was found that PEBA is a competitive inhibitor of chymotrypsin-catalyzed hydrolysis of methyl hippurate, with a dissociation

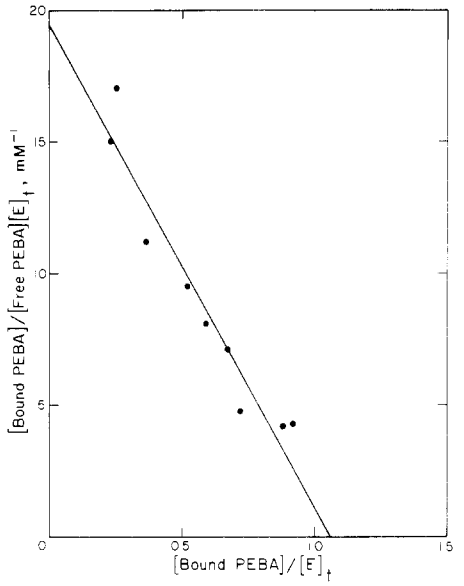


FIGURE 1: Binding of PEBA to chymotrypsin, at 25.0°. In each run, the total concentration of active chymotrypsin was 0.083 mM. The concentration of unbound PEBA, after equilibration, ranged from 0.015 to 0.21 mM. The buffer used for dialysis was 44 mM KCl–20 mM potassium phosphate (pH 7.4). The procedure is given in the Materials and Methods section. The data are plotted according to eq 1 in the text.

constant of 0.04 mM under the same conditions as those used for the equilibrium dialysis (Koehler and Lienhard, 1971).

Equilibrium dialysis was also performed with labeled PEBA and iPr_2P-chymotrypsin, under the conditions described in the legend of Figure 1. In one experiment, which was carried out in triplicate, the concentrations of iPr_2P-chymotrypsin and PEBA were 0.10 and 0.05 mM (after dialysis), respectively; in another, the concentrations were 1.0 and 1.9 mM (after dialysis), respectively. There was no detectable binding (less than 4% of the protein complexed with PEBA) in either case.

Binding of SBA to Chymotrypsin. SBA inhibits the chymotrypsin-catalyzed hydrolysis of methyl hippurate. Figure 2 presents the dependence of the reciprocal of the initial velocity upon the reciprocal of the substrate concentration in the presence and absence of SBA. The fact that the plots are linear and intersect on the $1/v$ axis shows that SBA is a competitive inhibitor (Dixon and Webb, 1964). The values of the dissociation constant for the enzyme–inhibitor complex given by these kinetic data are 0.022, 0.029, and 0.027 mM, at 0.013, 0.026, and 0.10 mM SBA, respectively.

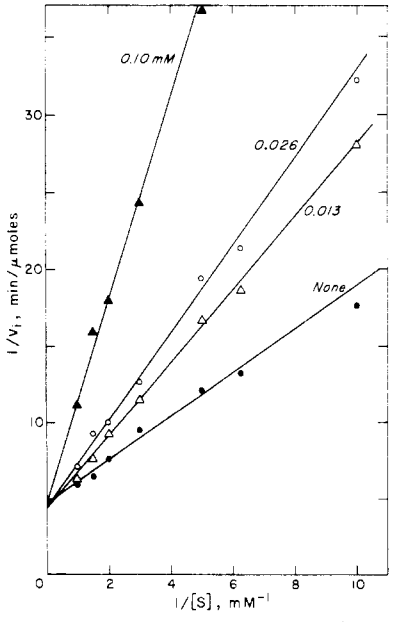


FIGURE 2: Inhibition of the chymotrypsin-catalyzed hydrolysis of methyl hippurate by SBA at pH 7.5 and 25.0°. The concentrations of SBA are given on the figure. Each point represents the average from duplicate determinations of the initial rates; the values from duplicate runs agreed with ±5% of the average.

TABLE I: Difference Absorbance at 265 nm upon Binding of SBA to Chymotrypsin.[a]

$[SBA]_t$ (mM)	$[EI]^b$ (mM)	$\Delta A_{265}{}^c$	$\Delta\epsilon_{265}{}^d$ (M^{-1} cm^{-1})
0.025	0.016	0.037	6000
		0.046	
0.050	0.029	0.086	6900
		0.089	
0.075	0.038	0.108	6300
0.100	0.044	0.117	6400
		0.130	
0.125	0.048	0.139	6700
		0.143	
0.150	0.051	0.153	
		0.174	7300
		0.168	

[a] Difference spectra were measured with SBA (compartment 1) and chymotrypsin (compartment 2) in the sample beam *vs.* SBA and chymotrypsin (compartment 1) and buffer (compartment 2) in the reference beam. The concentration of active chymotrypsin was 0.064 mM in each case. All solutions contained 44 mM KCl–20 mM potassium phosphate (pH 7.4). [b] See the text for the procedure used in calculating this quantity. [c] In most cases the final solutions were prepared from stock solutions several times, and the difference spectrum was measured with each preparation. The several entries for each $[SBA]_t$ are the individual values. [d] Average values, calculated according to eq 2.

The binding of SBA to chymotrypsin is accompanied by a spectral change in the region from 250 to 300 nm. The following analysis of this spectral change shows that it is consistent with the formation of a 1:1 complex between SBA and chymotrypsin, the dissociation constant for which is about 0.026 mM, the average of the kinetically determined values.

Difference spectra were measured with one concentration of the chymotrypsin and five concentrations of SBA, as described in Table I. The absorbance at any wavelength of the solutions in the sample beam (A_S) is equal to $\epsilon_E[E]_t l + \epsilon_{SBA}[SBA]_t l + A_0$, where ϵ_E and ϵ_{SBA} are the molar absorbancies of the enzyme and SBA, respectively; $[E]_t$ and $[SBA]_t$ are the total concentrations of active enzyme and SBA, respectively; l is the path length; and A_0 is the absorbance of the solvent. Similarly, the absorbance of the solutions in the reference beam (A_r) is equal to $\epsilon_E[E]l + \epsilon_{SBA}[SBA]l + \epsilon_{EI}[EI]l + A_0$, where [E], [SBA], and [EI] are the concentrations of the free enzyme, unbound SBA, and the SBA–enzyme complex, respectively, and ϵ_{EI} is the molar absorbancy of the SBA–enzyme complex. Since $[E]_t$ equals the sum of [E] and [EI] and $[SBA]_t$ equals the sum of [SBA] and [EI]

$$\epsilon_E + \epsilon_{SBA} - \epsilon_{EI} \equiv \Delta\epsilon = \frac{A_S - A_r}{[EI]l} \equiv \frac{\Delta A}{[EI]l} \quad (2)$$

Values of [EI] were calculated upon the assumption that the equation for a 1:1 complex with K_i equal to 0.026 mM holds

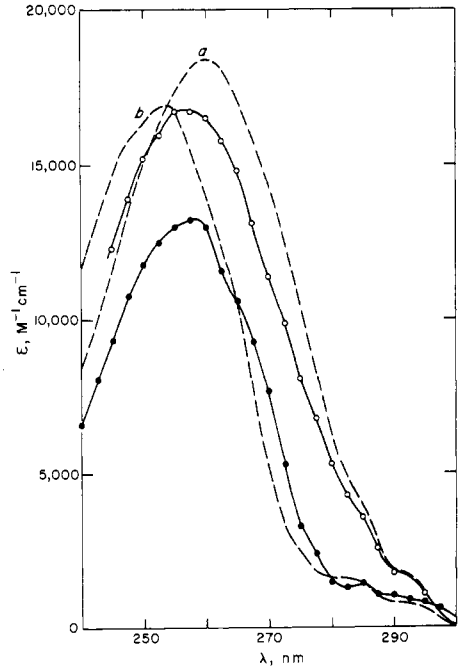


FIGURE 3: Spectra of SBA and its derivatives. The spectrum labeled *a* is that of SBA in 44 mM KCl–20 mM potassium phosphate (pH 7.5). Spectrum *b* is that of SBA in 0.10 N NaOH. The closed circles describe the difference spectrum of SBA–chymotrypsin *vs.* chymotrypsin in 44 mM KCl–20 mM potassium phosphate (pH 7.5). The open circles describe the spectrum of the adduct of *N*-methylimidazole with SBA.

(eq 3). These values were then used to compute values for $\Delta\epsilon$,

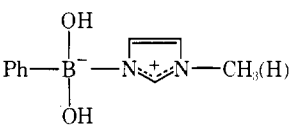
$$K_i = \frac{([E]_t - [EI])([SBA]_t - [EI])}{[EI]} \qquad (3)$$

according to eq 2. The validity of the assumption was shown by the finding that the values of $\Delta\epsilon$ at each wavelength were constant, within experimental error, over the range of computed [EI] values. Table I illustrates this result with the data at 265 nm, where there is a maximum in the difference spectra.

The values of $\Delta\epsilon$ at each wavelength were used to construct the difference spectrum of the SBA–chymotrypsin complex *vs.* chymotrypsin. This was done by subtracting $\Delta\epsilon$ from ϵ_{SBA} to give $\epsilon_{EI} - \epsilon_E$ (eq 2). The spectrum is shown in Figure 3. Since the binding of phenylalanine derivatives to the active site of chymotrypsin causes only small changes in the ultraviolet spectrum of the enzyme (the maximal value of $\Delta\epsilon$ is about 300 M^{-1} cm^{-1}) (Benmouyal and Trowbridge, 1966; Himoe *et al.*, 1967), this spectrum can be taken to be the spectrum of the bound SBA.

In contrast to the results with chymotrypsin, there was no difference spectrum generated with 0.067 mM SBA (compartment 1) and 0.10 mM iPr_2P-chymotrypsin (compartment 2) in the sample beam *vs.* 0.067 mM SBA–0.10 mM iPr_2P-chymotrypsin (compartment 1) and buffer (compartment 2) in the reference beam. All solutions here contained 44 mM KCl–20 mM potassium phosphate (pH 7.5).

Reaction of SBA with N-Methylimidazole. Previous work in this laboratory has revealed that in aqueous solution *N*-methylimidazole and imidazole add rapidly to the boron atom of benzeneboronic acid to give a compound of the structure



(Koehler *et al.*, 1972). The occurrence of the same type of

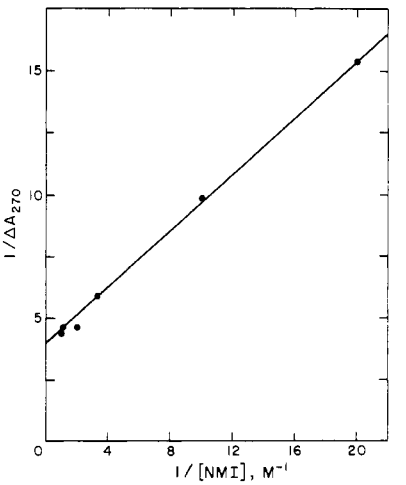


FIGURE 4: Adduct formation between SBA and *N*-methylimidazole. Difference spectra were measured with 0.20 mM SBA in 0.9 M KCl (compartment 1) and *N*-methylimidazole buffer–50% HCl (pH 7.35) (compartment 2) in the sample beam *vs.* 0.20 mM SBA–*N*-methylimidazole buffer–50% HCl (pH 7.35)–KCl to ionic strength 0.9 M (compartment 1) and H_2O (compartment 2) in the reference beam. The abcissa is the reciprocal of the concentration of the basic form of *N*-methylimidazole.

reaction with SBA was detected by difference spectroscopy. The equilibrium association constant (K_{Im}) for the reaction was determined by measuring the difference spectra between SBA in aqueous solution and SBA in *N*-methylimidazole buffers of various concentrations. It can be shown by a direct derivation that eq 4 describes the formation of a 1:1 complex, with

$$\frac{1}{\Delta A} = \frac{1}{K_{Im}\Delta\epsilon l[MeIm]} + \frac{1}{\Delta\epsilon[SBA]_t l} \qquad (4)$$

our method of measurement (see the legend of Figure 4). In this equation, ΔA is the difference absorbance, l is the path length of the cuvet compartment, and [MeIm] is the concentration of uncomplexed *N*-methylimidazole base, which here is virtually the same as the total concentration of *N*-methylimidazole base because *N*-methylimidazole was in great molar excess over SBA. $\Delta\epsilon$ formally equals the difference between the sum of the molar absorbancies of SBA and *N*-methylimidazole and that of the complex between the two (see eq 2). However, since the molar absorbancies of *N*-methylimidazole and of *N*-methylimidazolium are less than 1 M^{-1} cm^{-1} above 245 nm, $\Delta\epsilon$ is, in fact, simply equal to the difference between the molar absorbancies of SBA and the complex. Figure 4 presents the plot of the reciprocal of ΔA at 270 nm, where there is a maximum in the difference spectra, against the reciprocal of the concentration of *N*-methylimidazole. The value of K_{Im} is 6.6 M^{-1}, which is in the range of the value of 12 M^{-1} found previously for benzeneboronic acid. The value of $\Delta\epsilon$ at this wavelength is 2950 M^{-1} cm^{-1}. Values of $\Delta\epsilon$ at this and other wavelengths were used to construct the spectrum of the *N*-methylimidazole adduct (Figure 3).

Inhibition of Chymotrypsin by Aldehydes. The effects of hydrocinnamaldehyde and *trans*-cinnamaldehyde upon the rates of chymotrypsin-catalyzed hydrolysis of methyl hippurate were determined. Both compounds are competitive inhibitors. The value of K_i for hydrocinnamaldehyde is 0.38 mM at pH 7.8 (average of values of 0.49, 0.28, and 0.36 mM at 0.25, 0.50, and 0.93 mM inhibitor, respectively); that for *trans*-cinnamaldehyde is 2.6 mM at pH 7.8 (average of values of 2.4 and 2.8 mM at 0.50 and 2.0 mM inhibitor, respectively).

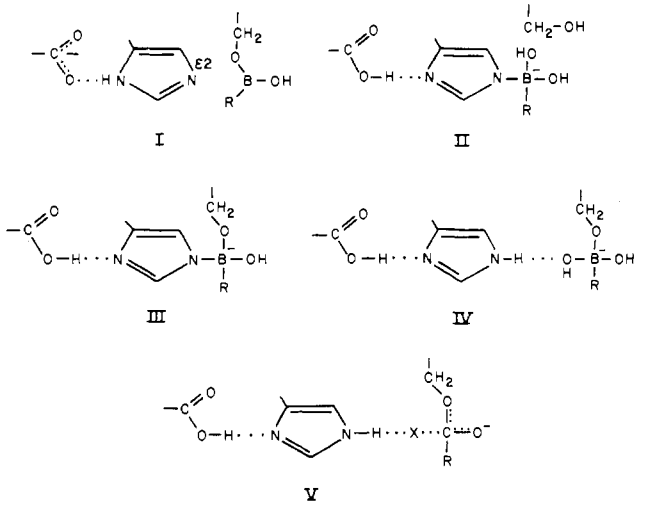


FIGURE 5: Schematic representations of possible structures for the complex of the active site of chymotrypsin with PEBA and SBA (I–IV) and for the tetrahedral-like transition state in the acylation or deacylation of chymotrypsin (V). The carboxyl, imidazole, and hydroxymethylene groups are from Asp-102, His-57, and Ser-195, respectively (Blow, 1971). R is $PhCH_2CH_2$ or PhCH=CH. In V, X is the leaving group for acylation and the nucleophile for deacylation. II–V show the proton between the carboxyl and imidazole to be located on the carboxyl. An alternate formulation is the one in which this proton is located on the imidazole. The decision to depict this proton on the carboxyl is based on the interpretation by Hunkapiller *et al.* (1973) of their nuclear magnetic resonance data for α-lytic protease. Hunkapiller *et al.* (1973) have concluded that the monoprotonated form of the carboxyl-imidazole pair at the active site of this serine protease is the neutral species (carboxylic acid–imidazole base) rather than the ion pair (carboxylate anion–imidazolium cation).

Discussion

The results from equilibrium dialysis and ultraviolet difference spectroscopy show that there is one binding site on chymotrypsin for PEBA and SBA. Since both compounds are competitive inhibitors and since neither compound binds to iPr_2P-chymotrypsin, this site is the active site. The unusual feature of these boronic acids is that they bind much more strongly than amides and esters of similar structure. The K_i for PEBA at pH 8 (0.04 mM) is smaller than that for hydrocinnamide (6 mM) by a factor of 150 (Koehler and Lienhard, 1971); similarly, the K_i for SBA at pH 7.5 (0.026 mM) is smaller than the dissociation constant of the complex between chymotrypsin and methyl *trans*-cinnamate (6.7 mM) (Bender and Zerner, 1962) by a factor of 250. Four structures that *a priori* might account for this tight binding are given in Figure 5. Structure I is a borate ester with the serine hydroxyl group at the active site. It is an analog of the acyl-enzyme intermediate that occurs in chymotrypsin-catalyzed acyl transfer reactions (Hess, 1971). Structures II and III are ones in which the imidazole group at the active site has added to the boron. In structure III the boronic acid is also esterified by the seryl hydroxyl group. Structure IV is analogous to the tetrahedral-like transition states through which acylation and deacylation of chymotrypsin are expected to proceed (Figure 5, structure V) (Jencks, 1969). Each structure has a net charge of −1. The pH dependence of the dissociation constant for PEBA (Koehler and Lienhard, 1971) requires this state of ionization in the complex. We will consider the evidence with regard to each structure in turn.

Structure I. It has previously been shown that the pH dependence of the dissociation constant for PEBA is not consistent with structure I (Koehler and Lienhard, 1971). The present results with SBA provide further evidence against structure I. The equilibrium constant for methanolysis of *trans*-cinnamoyl-chymotrypsin (eq 5) is equal to the ratio of the rate constant for methanolysis of the acyl-enzyme in aqueous solution to the rate constant for acylation of chymotrypsin with methyl *trans*-cinnamate (eq 6).

$$\mathrm{RCOCH_2E + MeOH \underset{k_E}{\overset{k_{MeOH}}{\rightleftharpoons}} RCOMe + HOCH_2E} \quad (5)$$

$$K_{MeOH} = \frac{[RCO_2Me][HOCH_2E]}{[RCO_2CH_2E][MeOH]} = \frac{k_{MeOH}}{k_E} \quad (6)$$

Bender and coworkers have reported values for each of these rate constants at 25° near pH 8 (Bender and Zerner, 1962; Bender *et al.*, 1964). The value of K_{MeOH} is 4 mM. If the equilibrium constant for the dissociation of the SBA-chymotrypsin complex is expressed in the analogous way by including the concentration of water (eq 7), its value is $0.025/55 = 4.7 \times 10^{-4}$ mM.

$$\mathrm{RBOCH_2E + HOH \rightleftharpoons RBOH + HOCH_2E}$$

$$K_i' = \frac{[RB(OH_2)][HOCH_2E]}{[RB(OH)OCH_2E][H_2O]} \quad (7)$$

Since the structures of the acyl-enzyme and the boronic acid ester (I) are similar and since the substitution of water for methanol would only change the value of the equilibrium constant for eq 5 by a factor of about three (Jencks and Gilchrist, 1964), the values of K_i' and K_{MeOH} would be expected to be approximately the same if I were the correct structure. The fact that they differ by a factor of 10^4 is evidence against I.

Structures II and III. The nonenzymatic addition of *N*-methylimidazole and imidazole to boronic acids, the equilibrium constants for which fall in the range of 2–12 M^{-1}, provide a model for structures II and III[2] (Koehler *et al.*, 1972; Philipp and Bender, 1971). Others have suggested that the potent inhibition of chymotrypsin and the similar protease, subtilisin, by benzeneboronic acid is due to either the formation of II (Philipp and Bender, 1971) or III (Antonov *et al.*, 1972). Recently, electron density difference maps of the crystalline complexes of subtilisin BPN′ with PEBA and with benzeneboronic acid *vs.* the enzyme itself at pH 7.5 have been determined by X-ray diffraction. In both cases the maps clearly show that the N-ε2 atom of the imidazole is located at least 4 Å from the boron atom, a distance which is too great for covalent bonding. The maps are most consistent with the conclusion that the structures are type IV (Birktoft, 1973). Since subtilisin BPN′ and chymotrypsin are similar in their catalytic properties and in the three-dimensional structures of their active sites (Kraut, 1971; Robertus *et al.*, 1972), it seems likely that the complexes with chymotrypsin also have structure IV.

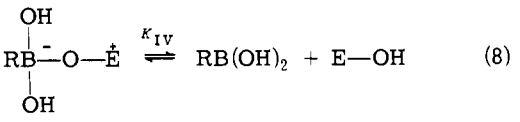
Structure IV. The acid dissociation constant of the carboxyl-imidazole pair at the active site of chymotrypsin is about 10^{-7} M (Hess, 1971), and the acid dissociation constant of a typical boronic acid is $10^{-9.5}$ M. Consequently, at first glance, structure IV, in which the acidic form of the carboxyl-imidazole pair is adjacent to the basic form of the boronic acid,[3] may

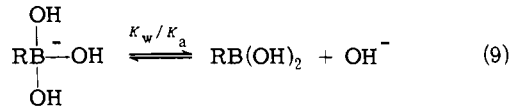
[2] The equilibrium constant for the addition of *N*-methylimidazole to PEBA at 30° has been determined by a pH perturbation method (unpublished data of K. Koehler and G. E. Lienhard) and by ^{11}B and ^1H nuclear magnetic resonance spectroscopy (unpublished data of B. Sykes and G. E. Lienhard). All three methods give a value of 2.5 M^{-1}.

[3] The weak acidity of boronic acids is due to the reaction $RB(OH)_2 + H_2O \rightarrow R\bar{B}(OH)_3 + H^+$ (Bell *et al.*, 1967).

seem an improbable structure for the complex. However, the following analysis shows that because of the resemblance of IV to the transition state and because of the tendency of alkoxide and hydroxide ions to add to boronic acids, the *estimated* values of the dissociation constants for type IV structures are substantially *smaller* than the observed values.

The equilibrium constant that we wish to estimate is that for the dissociation of IV (eq 8, K_{IV}). The value of the equilibrium constant for dissociation of the hydroxide ion adduct of a boronic acid (eq 9) is equal to the ion product of water (K_w) divided by the acid dissociation constant of the boronic acid (K_a).[3] Equilibria that are analogous to those of eq 8 and 9 are the dissociation of the tetrahedral-like transition state for acylation of chymotrypsin by a methyl ester (V) and the dissociation of the tetrahedral-like transition state for reaction of the ester with hydroxide ion, to re-form the reactants (eq 10 and


(8)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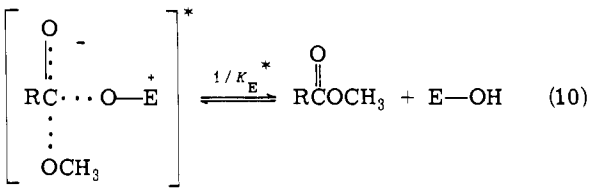
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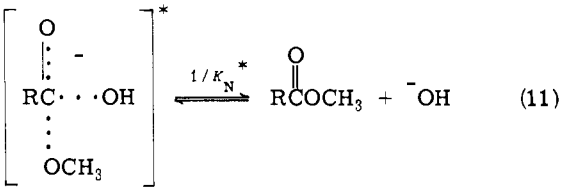
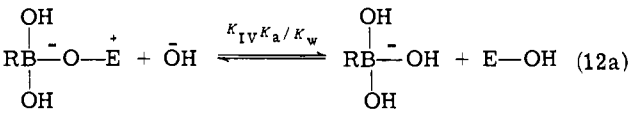
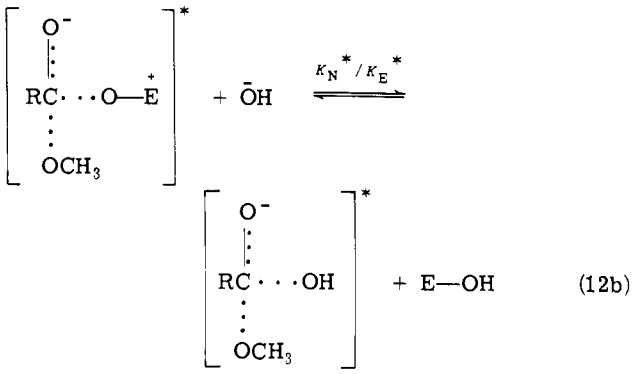

(11)

11, respectively). Combination of the equilibria in eq 8 and 9 and those in eq 10 and 11 lead to the overall equilibria


(12a)


(12b)

The magnitude of the ratio of the equilibrium constant for eq 12a to that for eq 12b will depend on the extent to which the stability of IV relative to that of $R\bar{B}(OH)_3$ is the same as the stability of V relative to that of the transition state for alkaline hydrolysis. It seems likely that these relative stabilities are approximately the same, since IV and $R\bar{B}(OH)_3$ are similar in structure to V and the transition state for alkaline hydrolysis, respectively, and also since each equilibrium involves only the interconversion of tetrahedral structures. Consequently, as an approximation

$$K_{IV}K_a/K_w = K_N^*/K_E^* \qquad K_{IV} = K_wK_N^*/K_aK_E^* \tag{13}$$

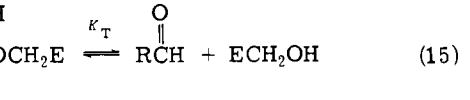
According to the transition-state theory of reaction rates (Laidler, 1969), K_N^* is directly proportional to the second-order rate constant for reaction of the ester with hydroxide ion (k_N) and K_E^* is directly proportional to the second-order rate constant for acylation of chymotrypsin by the ester (k_E). Since the proportionality constant is the same in both cases, eq 13 becomes

$$K_{IV} = K_wk_N/K_ak_E \tag{14}$$

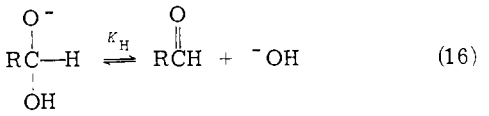
Thus, the smaller the value of the constant for dissociation of hydroxide ion from the boronate anion (K_w/K_a) and the larger the rate of acylation of the enzyme relative to the rate of reaction with hydroxide ion (k_E/k_N), the smaller is the value predicted for K_{IV}. In the case of SBA, the value of K_w/K_a at 25° is 4.3×10^{-2} mM, and the value of the ratio of the second-order rate constant for hydroxide ion catalyzed hydrolysis of methyl *trans*-cinnamate (k_N) to that for acylation of chymotrypsin by this ester (k_E) at 25° is 0.017 (Bender and Zerner, 1962). Thus, the value of K_{IV} is 7×10^{-4} mM. A similar calculation for PEBA has given the value of 9×10^{-5} mM (Koehler and Lienhard, 1971). These values are smaller than the measured dissociation constants by a factor of 40 for SBA and 450 for PEBA.

It was our hope that a comparison of the ultraviolet spectrum of SBA bound to chymotrypsin with the spectra of SBA (a model for structure I), the *N*-methylimidazole adduct of SBA (a model for II and III), and the basic species of SBA (a model for IV) would provide information about the structure of enzyme complex in solution. However, the spectrum of the enzyme-bound SBA (λ_{max} 257 (ϵ 13,300 M^{-1} cm^{-1})) is different from the spectra of SBA (λ_{max} 260 (ϵ 18,400 M^{-1} cm^{-1})), the *N*-methylimidazole adduct (λ_{max} 257 (ϵ 16,700 M^{-1} cm^{-1})), and the hydroxide ion adduct (λ_{max} 254 (ϵ 16,800 M^{-1} cm^{-1})). In this regard, it is noteworthy that the ultraviolet spectrum of the *trans*-cinnamoyl group in ester linkage to seryl residue 195 of native chymotrypsin (λ_{max} 292 (ϵ 17,000 M^{-1} cm^{-1})) is substantially different from that of the model compound, *trans*-cinnamoyl-*N*-acetylserinamide (λ_{max} 281 (ϵ 24000 M^{-1} cm^{-1})) (Bender *et al.*, 1962; Bender and Kezdy, 1965; Mercouroff and Hess, 1963; Bernhard *et al.*, 1965). These data for the esters show that interaction of the PhCH=CHC(=O) group with chymotrypsin causes a shift in λ_{max} to a longer wavelength and a decrease in ϵ_{max}. The tenuous generalization of this type of spectral perturbation to the PhCH=CHB(OH) group leads to the conclusion that the spectrum of SBA bound to chymotrypsin is most consistent with structure IV.

Aldehydes derived from specific substrates have been found to be very potent competitive inhibitors of serine and cysteine proteases (Umezawa, 1972; Tatsuta *et al.*, 1973; Ito *et al.*, 1972; Westerik and Wolfenden, 1972; Thompson, 1973). This inhibition may be due to the formation of a hemiacetal or hemimercaptal from the seryl or cysteinyl residue at the active site (eq 15, for serine proteases) (Westerik and Wolfenden, 1972;


(15)

Thompson, 1973). Values of the dissociation constant for eq 15 (K_T) can be estimated in a way similar to that described above for the boronic acids. The relevant equilibria are the dissociation of hydroxide ion from the alkoxide ion of the aldehyde hydrate (eq 16) and the dissociations of the transition state for



(16)

acylation of the enzyme (eq 10) and for reaction of the methyl ester with hydroxide ion (eq 11). Thus, to the extent that the stability of the hemiacetal complex with the enzyme relative to the stability of the aldehyde hydrate anion is the same as the stability of V relative to the transition for alkaline hydrolysis of the ester, eq 17 holds. In the case of hydrocinnamaldehyde, the

$$K_T = K_H (k_N/k_E) \quad (17)$$

value of K_H should be about the same as the value for acetaldehyde, which is 0.2 M (Bell, 1966), and the value of k_N/k_E is 10^{-3} (Koehler and Lienhard, 1971). Thus, an estimate of K_T is 0.2 mM, which is remarkably close to the observed value of 0.38 mM. This close agreement is probably fortuitous in the sense that there are evident differences as well as similarities between the structures of the hemiacetal complex and V and between the structures of the aldehyde hydrate anion and the transition state for alkaline hydrolysis of the ester. In the case of *trans*-cinnamaldehyde, the value of K_H is not known but is probably greater than 0.2 M, since the tendency of hydrocinnamaldehyde to form an adduct is greater than that of *trans*-cinnamaldehyde.[4] The value of k_N/k_E is 0.017. Thus, we estimate that K_T is greater than 3.4 mM. The observed value is 2.6 mM. These values are in the same range as that associated with the noncovalent binding of methyl *trans*-cinnamate (dissociation constant 6.7 mM). Thus, it seems likely that a significant fraction of the complex of chymotrypsin with *trans*-cinnamaldehyde may have a structure in which the serine hydroxyl is not added to the carbonyl group.

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[4] The fact that the molar absorbancy of *trans*-cinnamaldehyde at λ_{max} in water (λ_{max} 291 (ϵ 24,200 M^{-1} cm^{-1})) is slightly larger than that in hexane (λ_{max} 280 (ϵ 21,200 M^{-1} cm^{-1})) indicates that in water the compound is less than 10% in the form of the hydrate. α,β-Saturated aldehydes like hydrocinnamaldehyde are about 60% hydrated in water (Bell, 1966).