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SUBSTITUENT EFFECT ON THE ELEMENTARY PROCESSES OF THE INTERACTION BETWEEN SEVERAL BENZENEBORONIC ACIDS AND SUBTILISIN BPN'

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Summary

Benzeneboronic acid, a transition-state analog for serine proteases, binds to the catalytic center of subtilisin BPN'. The binding mechanism is so-called two-step mechanism; the initial fast association followed by a slow unimolecular process (Nakatani, H., Uehara, Y. and Hiromi, K. (1975) J. Biochem. (Tokyo) 77, 615—616),

$$E + S \overset{\text{fast}}{\rightleftharpoons} ES \overset{\text{slow}}{\rightleftharpoons} ES^*$$

(E = subtilisin, S = benzeneboronic acid).

The structure of the transient complex (ES) at the initial association process was manifested by the substituent effect of benzeneboronic acid on the rate parameters in the elementary processes. The study by the temperature-jump and stopped-flow methods showed that the boron atom in benzeneboronic acid strongly interacts with a nucleophilic site, probably, O_γ of Ser-221 or imidazole of His-64 at the catalytic center, already at the initial fast association.

Introduction

So-called transition-state analogs for serine proteases, such as alkyl and aryl boronic acids, have been considered to bind to the catalytic serine residue forming tetrahedral adducts [1—6]. Recent X-ray crystallographic study on the subtilisin-benzeneboronic acid complex showed that benzeneboronic acid actually forms a tetrahed adduct with O_γ of Ser-221 [7]. Our recent studies by the temperature-jump relaxation method on the subtilisin-benzeneboronic acid system showed that the binding process is not a simple bimolecular association [8,9]. The most probable mechanism was a two-step mechanism; a fast bimolecular association followed by a slow unimolecular isomerization process,

$$E + S \overset{K_1}{\rightleftharpoons} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES^* \qquad (1)$$

where E and S are the enzyme and benzeneboronic acid, ES and ES* are structural isomers of subtilisin-benzeneboronic acid complexes, K_1 is the association constant for the fast association process, k_2 and k_{-2} are rate constants for the isomerization reaction. The values of k_2 and k_{-2} are essentially pH-independent but K_1 is pH dependent; neutral benzeneboronic acid binds to the catalytic site with deprotonated His 64 of subtilisin, forming ES [9,10]. We proposed that the isomerization process, ES $\rightleftharpoons$ ES*, is a trigonal-tetrahedral interconversion of benzeneboronic acid at the catalytic site of the enzyme. Although ES* is most reasonably considered to be a tetrahedral adduct as has been clarified by X-ray crystallographic analysis [7], no structural information about the transient complex, ES, has been obtained. In this paper, we used substituted benzeneboronic acids since the information from Hammett relation on the rate parameters, K_1, k_2 and k_{-2} would be useful for elucidating rational reaction mechanism.

Experimental

Materials. Crystalline subtilisin BPN' was purchased from Nagase Sangyo Co. Ltd. The active site concentration was determined from burst titration with N-*trans*-cinnamoylimidazole [11]. Benzeneboronic acid was purchased from Sigma and recrystallyzed twice from hot water. *m*-Nitrobenzeneboronic acid and *p*-bromo-benzeneboronic acid were purchased from Pfaltz and Bauer. *m*-Chloro-benzeneboronic acid was kindly supplied by Dr. Okuyama of Osaka University. Thin-layer chromatograms for substituted benzeneboronic acids with chloroform/ether (3 : 2) showed single spots.

Method. Inhibition constants of the boronic acids to subtilisin were determined with a Radiometer TTT2b pH-stat using *N*-acetyltyrosine ethyl ester as a substrate. The pK value for His-64 of subtilisin was determined from pH-dependence of the inhibitor constant of benzeneboronic acid at 15°C. The pK values of the boronic acids were determined by the spectrophotometric titration using Union-Giken SM-401 spectrophotometer at 15°C. For H-, *m*- chloro-,

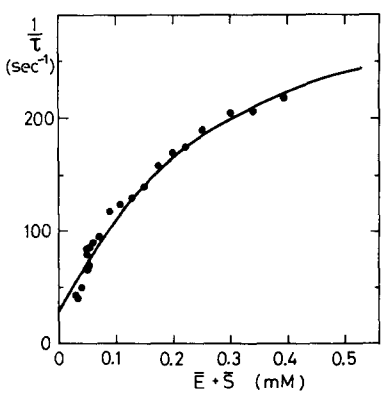


Fig. 1. Concentration dependence of reciprocal relaxation time ($1/\tau$) in *m*-nitrobenzeneboronic acid-subtilisin system at pH 6.5 and 15°C. ($\bar{E} + \bar{S}$) represents sum of equilibrium concentration of *m*-nitrobenzeneboronic acid and subtilisin.

and *p*-bromobenzeneboronic acid-subtilisin systems, kinetic experiments were carried out with a temperature-jump apparatus (Union-Giken RA-105) using a pH indicator, *p*-nitrophenol, in 0.2 M KNO_3 at pH 6.5; the experimental procedures were described elsewhere [8,9]. The stopped-flow method (Union-Giken SF-70) was used for the kinetic study on the *m*-nitrobenzeneboronic acid-subtilisin system. The binding process was measured directly by the appearance of difference absorbance at 300—320 nm [12]. The concentration dependence of reciprocal relaxation time at pH 6.5 and 15°C is shown in Fig. 1. Rate parameters, K_1, k_2, and k_{-2} for each system were determined from the analysis of the concentration dependence of reciprocal relaxation time [8,9].

Results

In our previous papers on the benzeneboronic acid-subtilisin system, it was shown that the value of log K_1 depends linearly on pH with slope of unity, whereas the values of log k_2, log k_{-2} and log k_2/k_{-2} are independent of pH below pH 7; the values are 3.30 to 3.10, 3.13 to 2.92 and 0.42 to —0.01, respectively, between pH 5.5 and 6.9 at 25°C [9,10].

The overall association constant, K_a, obtained from titrations also depends linearly on pH with slope of unity below pH 7, i.e. only deprotonated form of His-64 of the enzyme binds with neutral benzeneboronic acid [3,9,10]. The pH dependence of K_a and K_1 are expressed as follows:

$$K_a = \frac{K_a^\circ}{(1 + (H^+)/K_E)(1 + K_B/(H^+))} \tag{2}$$

$$K_a^\circ = K_1^\circ \left(1 + \frac{k_2}{k_{-2}}\right) \tag{3}$$

where K_a° and K_1° are pH independent association constants. K_E and K_B are dissociation constants of proton equilibria for the enzyme (His-64) and benzeneboronic acid, respectively. The association constant of benzeneboronic acid with OH^-, K_{OH}, is obtained from the value of K_B:

$$K_{OH} = \frac{(\text{R-B(OH)}_3^-)}{(\text{R-B(OH)}_2)(OH^-)} = 10^{14.24} K_B \tag{4}$$

The value of pK_E was determined to be 7.39 at 15°C from pH dependence of K_a in benzeneboronic acid-subtilisin system as described in experimental section. The values of K_a for substituted benzeneboronic acid-subtilisin systems were determined as reciprocal inhibitor constants at pH 6.5, and the values of K_a° were calculated using Eqn. 2. Rate parameters K_1, k_2, and k_{-2} were obtained from the analysis of the temperature-jump or stopped-flow experiments at pH 6.5.

The values of K_1° were calculated using Eqn. 3. Whole numerical data including Hammett's σ values for substituent benzeneboronic acids are listed in Table I. The general expression of Hammett's rule is shown as follows,

$$\log \frac{k}{k_o} = \rho\sigma \tag{5}$$

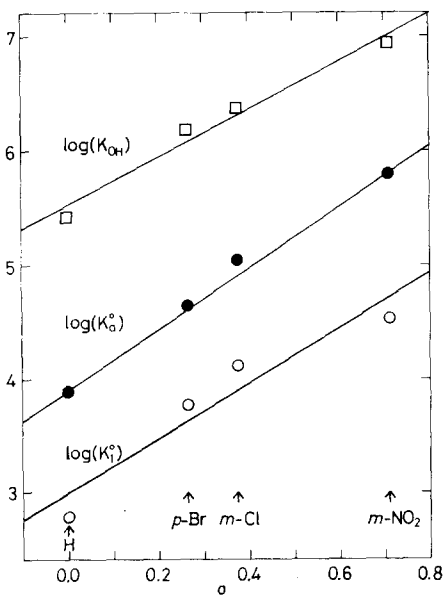
TABLE I

RATE PARAMETERS FOR SUBSTITUTED BENZENEBORONIC ACIDS AT 15°C

The values of K_a, K_1, k_2, and k_{-2} are determined at pH 6.50. The values of σ are cited from literature [12].

	pK_B	K_a (10^3 M^{-1})	K_a^0 (10^3 M^{-1})	K_1 (10^3 M^{-1})	K_1^0 (10^3 M^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})	$\frac{k_2}{k_{-2}}$	σ
H^-	8.80	0.794	6.19	0.0776	0.601	2420	262	9.24	0
p-Br^-	8.06	5.54	44.2	0.741	5.91	1516	234	6.48	0.265
m-Cl^-	7.87	13.6	110	1.47	13.0	319	38.9	8.20	0.373
m-NO_2^-	7.29	53.9	486	3.73	33.6	332	24.7	13.4	0.710

where k refers to the rate or equilibrium constant of a reaction and k_0 refers to the reference compound, unsubstituent compound. σ is the substituent constant of the substituted compound defined basically as $\sigma = \log (K/K_0)$ where K is the acid dissociation constant of the substituted benzoic acid and K_0 that of benzoic acid itself. ρ is the reaction constant for the reaction system. Fig. 2 shows the plots of log K_{OH}, log K_a^0, and log K_1^0 vs. σ. The each plot follows Hammett's law with $\rho = 2.1 \pm 0.2$, 2.7 ± 0.3, and 2.4 ± 0.5 for log K_{OH}, log K_a, and log K_1, respectively. Fig. 3 shows plot of log k_2, log k_{-2}, and log (k_2/k_{-2}) vs. σ. Both log k_2 and log k_{-2} decrease with increasing σ values: the ρ values are



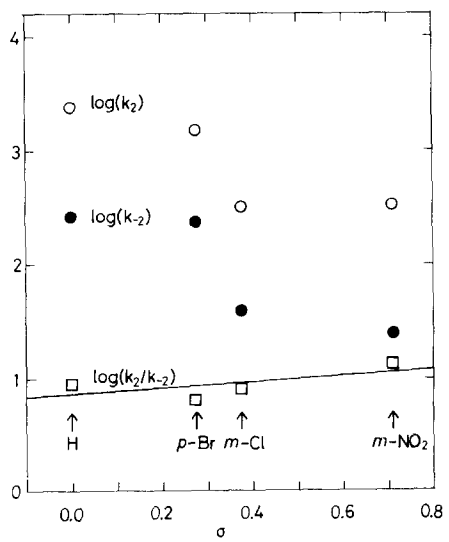


Fig. 2. Hammett relationship of equilibrium constants, K_{OH}, K_a^0, and K_1^0 (Eqns. 2 and 3). The σ values for substituted benzeneboronic acids are cited from literature [12]. The straight lines are obtained by the least-squares method.

Fig. 3. Plots of rate parameters on unimolecular process (Eqn. 1) vs. σ values of substituted benzeneboronic acids. The straight line for log (k_2/k_{-2}) is obtained by the least-squares method.

-1.3 ± 0.6 and -1.6 ± 0.6 for log k_2 and log k_{-2}, respectively. The value of log (k_2/k_{-2}) is nearly independent on σ values: the ρ values is 0.26 ± 0.26.

Discussion

As shown in Fig. 2, the slopes of the plots of log K_{OH}, log K_a^o, and log K_1^o vs. σ are nearly the same; the values of ρ are 2.1—2.7. The literature values of ρ for log K_{OH} are 2.00 [14] or 2.146 (Kajimoto, O., Saeki, T., Nagaoka, Y. and Fueno, T., manuscript in preparation) which well coincide with our result. Substituent groups which withdraw electron from boron atom of benzeneboronic acid will strengthen B-O bond of both R-B $(OH)_4^-$ and the tetrahedral adduct, ES* in Eqn. 1, in a similar manner. The results in Figs. 2 and 3 show that the transient complex ES in Eqn. 1 is also stabilized by the substituent effect, suggesting the interaction between boron atom of benzeneboronic acid and nucleophilic site of subtilisin. Two possible reaction sites which may interact with boron atom of benzeneboronic acid exist in the active site of subtilisin; a basic nitrogen atom of His-64 and alcoholic oxygen atom of Ser-221. In our previous paper [12], it is shown that the pH-independent association constant of *m*-nitrobenzeneboronic acid with *N*-methylimidazole is about twice as large as that of benzeneboronic acid with *N*-methylimidazole, which may show that both B-N and B-O interactions are strengthen by *m*-nitro-substituent. Kajimoto et al. studied binding process between substituted benzeneboronic acid and OH^- (Eqn. 3) by the temperature-jump method (Kajimoto, O., Saeki, T., Nagaoka, Y. and Fueno, T., manuscript in preparation). The reaction mechanism was analyzed as a simple bimolecular association and the values of formation rate constants were of the order of 10^7—10^8 $M^{-1} \cdot s^{-1}$ at 35°C.

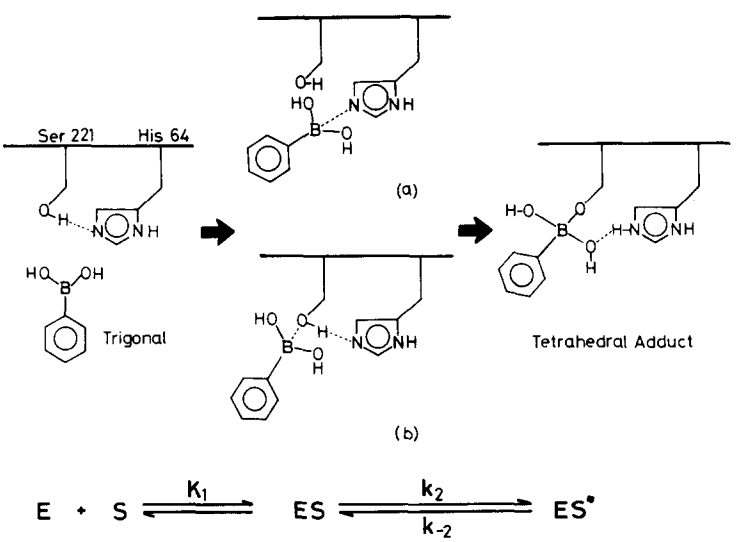


Fig. 4. Possible binding models for the transient complex, ES, in Eqn. 1 and the binding mechanism: (a) the imidazole complex, (b) the serine complex. In both B-N and B-O interactions, the binding mode is considered to be noncovalent.

The rate constants for the formation reaction obeyed the Hammett relationship with $\rho = 1.18$ at 35°C. Since the formation reaction rate constants are 2—3 orders smaller than the ordinary diffusion-controlled reaction [15], the rate determining process may be a chemical process; most reasonably, the conversion of benzeneboronic acid from trigonal to tetrahedral structure.

The sign of ρ is plus for log K_1 and minus for log k_2 and log k_{-2}, therefore, the more ES complex is stabilized, the slower becomes the rate of the interconversion between ES and ES*. To interpret rationally above results, two reaction mechanisms for the elementary processes in the benzeneboronic acid-subtilisin system are possible. Fig. 4 shows the proposed two mechanisms. In the transient complex, ES, boron atom of benzeneboronic acid interacts with imidazole of His-64 or oxygen atom of Ser-221; probably benzeneboronic acid is still a trigonal form. The conformation of the trigonal form is then transformed into a tetrahedral structure in ES* as shown in Fig. 4.

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