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BINDING OF *m*-NITROBENZENEBORONIC ACID TO THE ACTIVE SITE OF SUBTILISIN BPN'

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Summary

m-Nitrobenzeneboronic acid as a possible transition-state analog for serine proteases was found to cause absorption spectral change from 250 nm to 350 nm upon binding with subtilisin BPN' (EC 3.4.21.14) at pH 6.5. Similar difference spectral changes of *m*-nitrobenzeneboronic acid were also observed at alkaline pH or upon addition of *N*-methylimidazole at pH 6.5.

A characteristic circular dichroism spectrum of *m*-nitrobenezeneboronic acid was induced upon binding with subtilisin BPN' not only at pH 6.5, but also at alkaline pH. Circular dichroism spectral titration confirmed the stoichiometry of 1 : 1 for the *m*-nitrobenzeneboronic acid · subtilisin complex. *m*-Nitrobenzeneboronic acid was shown to be useful as a reversible chromophoric probe for the catalytic site of serine proteases.

Introduction

Alkyl and aryl boronic acids or aldehydes are considered transition-state analogs for serine proteases, because they can form tetrahedral adducts with O_γ of the serine residue at the catalytic site [1—6]. The proposed structure of the transition state analog · enzyme complexes resembles the transition state for acylation or deacylation process in serine protease-catalyzed reactions [7]. Crystallographic studies on the benzeneboronic acid · subtilisin complex proved that benzeneboronic acid yields a tetrahedral adduct at the catalytic site of the enzyme forming a covalent bond between boron and O_γ of Ser 221 [8].

When benzeneboronic acid was bound to subtilisin (EC 3.4.21.14) below pH 7.1, proton release from His 64 was observed [9]. By utilizing this phenomenon, the binding process between benzeneboronic acid and subtilisin was studied by the temperature-jump method using pH indicators [10]. In this paper, we studied the interaction between the transition state analog and subtilisin by

using *m*-nitrobenzeneboronic acid. The introduction of a nitro group to benzeneboronic acid enabled direct spectroscopic measurement in the near ultraviolet/visible region.

Experimental

Crystalline subtilisin BPN′ was purchased from Nagase Sangyo Co., Ltd. The active site concentration was determined from burst titration with *N-trans*-cinnamoyl imidazole [11]. *m*-Nitrobenzeneboronic acid was purchased from Pfaltz and Bauer Inc.; a thin-layer chromatogram with $CHCl_3$/diethylether (3 : 2, v/v) showed a single spot. The p*K* value of *m*-nitrobenzeneboronic acid was determined to be 7.2 at 25°C by spectrophotometric titration. The enzymatic reaction and its inhibition by *m*-nitrobenzeneboronic acid were measured with a Radiometer TTT2b pH stat using *N*-acetyltyrosine ethyl ester as a substrate at ionic strength of 0.2 M (KNO_3).

Absorption difference spectra were measured with Union-Giken SM 401 spectrophotometer. Circular dichroism spectra were measured with Jovin-Ivon-Dichro Graph Mark-III J. The enzyme concentration was 0.0322 mM for the absorption spectral titration and 0.700 mM for circular dichroism spectral titration.

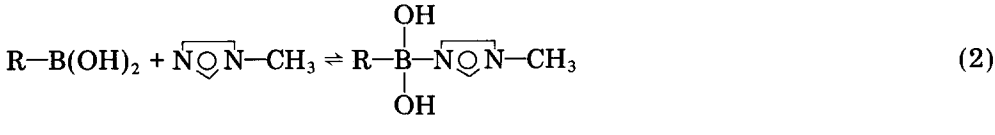
Results

Difference spectra

The absorption maximum of *m*-nitrobenzeneboronic acid shifts to longer wavelength (from 271 nm to 283 nm) as the pH was raised from 6.5 to the alkaline region. This was due to the addition of hydroxide to boron atom of the boronic acid [5], which is converted from trigonal to tetrahedral configuration.

$$R{-}B(OH)_2 + OH^- \rightleftharpoons R{-}B(OH)_3^- \qquad (1)$$

Similar spectral shifts of *m*-nitrobenzeneboronic acid were observed in the presence of *N*-methylimidazole or the enzyme, subtilisin, at pH 6.5 and these can be attributed to the formation of tetrahedral complexes as follows:



$$R{-}B(OH)_2 + \underset{\text{(Ser 221)}}{HO{-}E} \rightleftharpoons R{-}\overset{\overset{OH}{|}}{\underset{\underset{OH}{|}}{B}}{-}O{-}E \qquad (3)$$

The association constant for 1 : 1 adduct between *m*-nitrobenzeneboronic acid or *N*-methylimidazole was determined by a difference spectrophotometric titration [12]. The values of association constant, K_N, and molar absorptivity difference, $\Delta\epsilon_N$, at 300 nm were 97.1 M^{-1} and 1380 $M^{-1} \cdot cm^{-1}$, respectively, at pH 6.5.

Binding of *m*-nitrobenzeneboronic acid with subtilisin was also studied by difference spectrophotometric titration according to the following scheme:

$$E + I \overset{K_a}{\rightleftharpoons} EI \tag{4}$$

$$\Delta A_E = \Delta\epsilon_E(EI) \tag{5}$$

where E, I and EI represent the enzyme, *m*-nitrobenzeneboronic acid, and the enzyme · *m*-nitrobenzeneboronic acid complex, respectively. ΔA_E and $\Delta\epsilon_E$ are the absorbance difference and the molar absorptivity difference of the complex, respectively. The concentration of the complex is shown as (*EI*). The association constant K_a and $\Delta\epsilon_E$ at 300 nm were determined using the following relation:

$$\Delta A_E = \frac{\Delta\epsilon_E}{2}\left[(E_0 + I_0 + 1/K_a) - \sqrt{(E_0 + I_0 + 1/K_a)^2 - 4E_0I_0}\right] \tag{6}$$

where E_0 (0.0232 mM) and I_0 (0.01 mM—0.30 mM) are the analytical concentrations of the enzyme and *m*-nitrobenzeneboronic acid, respectively. The values of K_a and $\Delta\epsilon_E$ at 300 nm were estimated by the least-squares method to be $2.0 \cdot 10^4$ M^{-1} and 1670 $M^{-1} \cdot cm^{-1}$, respectively, at pH 6.5 and 25°C. The value of $\Delta\epsilon_E$ decreased in the higher pH region. The value of reciprocal inhibitor constant for *m*-nitrobenzeneboronic acid using *N*-acetyl tyrosine ethyl ester as a substrate was determined to be $2.8 \cdot 10^4$ M^{-1} at pH 6.5, which is in reasonable agreement with the value of K_a at pH 6.5.

Thr molar absorptivity difference due to the formation of adducts of *m*-nitrobenzeneboronic acid with *N*-methylimidazole or subtilisin was determined in this manner at each wavelength. The difference spectra thus constructed are shown in Fig. 1, together with the pH difference spectrum of *m*-nitrobenzeneboronic acid at pH 12 using pH 6.5 as reference. It is noticed that the three spectra are very similar.

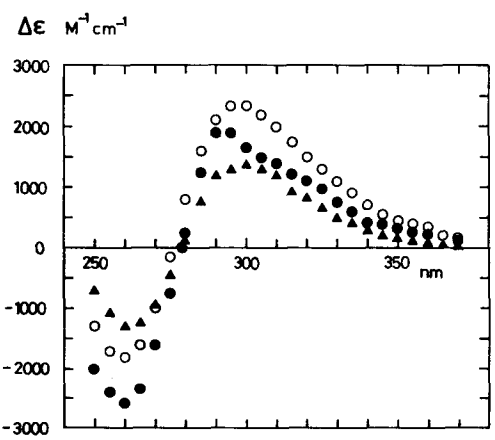


Fig. 1. Molar absorptivity difference spectra of *m*-nitrobenzeneboronic acid caused by its interaction with OH^- (at pH 12), *N*-methylimidazole and subtilisin (at pH 6.5): ○———○, R-B $(OH)_3^-$; ▲———▲, adduct with *N*-methylimidazole; ●———●, adduct with subtilisin. The reference solution for the complex of *m*-nitrobenzeneboronic acid with *N*-methylimidazole or subtilisin contains unmixed components in the separated cuvettes.

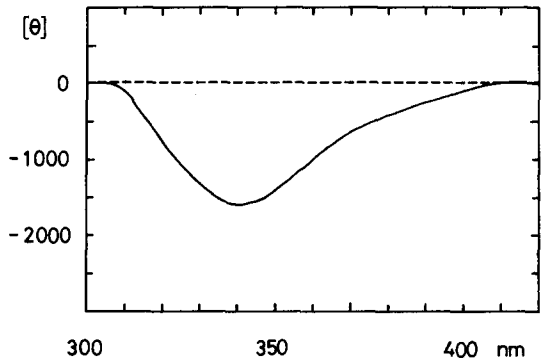


Fig. 2. Induced circular dichroism spectrum of *m*-nitrobenzeneboronic acid caused by its interaction with subtilisin at pH 6.5. [θ] is the molar ellipiticity and the dashed line is a baseline.

Circular dichroism spectrum and stoichiometry of binding

A characteristic negative circular dichroism spectrum of *m*-nitrobenzeneboronic acid was induced upon binding with subtilisin at near ultraviolet region as shown in Fig. 2, whereas no circular dichroism spectrum was observed for free *m*-nitrobenzeneboronic acid at any pH or for its complex with *N*-methylimidazole. The shape and intensity of the induced circular dichroism spectrum of *m*-nitrobenzeneboronic acid bound to subtilisin did not depend on pH. The asymmetry factor, *g*, which is defined as the ratio of $\epsilon_L - \epsilon_R (\epsilon_L - \epsilon_R = [\theta]/3300$; θ is the molar ellipticity) to the molar absorptivity, was $7.5 \cdot 10^{-4}$ at 340 nm. Since the intensity of the induced circular dichroism spectrum of *m*-nitrobenzeneboronic acid should be proportional to the concentration of the complex, the circular spectral titration at 350 nm was carried out to confirm the stoichiometry of binding. *m*-Nitrobenzeneboronic acid in methanol was added to the enzyme solution with a microsyringe. Fig. 3 shows the plot of $|A_L - A_R|$ (the amplitude of the negative circular dichroism band at 350 nm) vs. *I/E* expressed by the ratio of analytical concentrations of *m*-nitrobenzene-

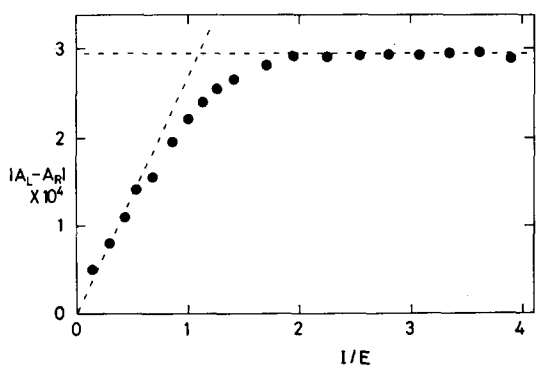


Fig. 3. The relationship between the amplitude of the negative circular dichroism band at 350 nm and the *m*-nitrobenzeneboronic acid/subtilisin ratio at pH 6.5. The enzyme concentration is fixed (0.7 mM) and the concentration of *m*-nitrobenzeneboronic acid is varied. The intersection of the initial slope and the saturation line shows the approximate equivalence point of 1 : 1.

boronic acid and subtilisin. Since $E_0K_a = 14$, this plot may suggest the stoichiometry of binding [13]. The intersection between the initial slope and the saturation line is at approx. 1.

Discussion

Neutral benzeneboronic acid binds only the deportonated form of *N*-methylimidazole [12]. Since the pK values for *m*-nitrobenzeneboronic acid and *N*-methylimidazole are 7.20 and 7.34, respectively, the value of the pH-independent association constant (K_N°) for the *m*-nitrobenzeneboronic acid · *N*-methylimidazole complex was calculated to be 875 M^{-1} at 25°C, using the following equation [12];

$$K_N = \frac{K_N^\circ}{\left(1 + \frac{(H^+)}{K_{IM}}\right)\left(1 + \frac{K_B}{(H^+)}\right)} \quad (7)$$

where K_{IM} and K_B are the dissociation constants of proton equilibria for *N*-methylimidazole and *m*-nitrobenzeneboronic acid, respectively. The values of pK of non-substituted benzeneboronic acid and pH-independent association constant with *N*-methylimidazole are 8.86 and 10.7 M^{-1} at 25°C [12]. Therefore it seems that the substituted nitro group makes the boron atom more electrophilic and stabilizes the adduct with OH^- or *N*-methylimidazole. The difference spectra of *m*-nitrobenzeneboronic acid complexes with OH^-, *N*-methylimidazole and subtilisin are very similar: the spectral shift of neutral *m*-nitrobenzeneboronic acid may result from the interaction with any suitable nucleophilic sites. However, the appearance of the induced circular dichroism spectrum of *m*-nitrobenzeneboronic acid upon binding with subtilisin indicates the asymmetric field of the catalytic site of the enzyme subtilisin. Our preliminary experiments showed that similar circular dichroism spectra are observed in the complexes with *Aspergillus melleus* semi-alkaline protease [6,14,15] and α-chymotrypsin. Thus the present study has shown that *m*-nitrobenzeneboronic acid (like some aryl arsenates [16]) is useful as a reversible chromophoric probe for the catalytic site of serine proteases.

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