INHIBITION OF SUBTILISIN BY SUBSTITUTED ARYLBORONIC ACIDS

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1. Introduction

Alkyl and arylboronic acids are potent competitive inhibitors of serine proteases [1–5]. These compounds have been known to inhibit hydrolytic enzymes at least since Torssel showed that benzeneboronic acids are potent inhibitors of serum cholinesterase [6]. Crystal structure determinations [7], NMR studies [8], and laser Raman studies [9] have helped to determine the structure of the enzyme–inhibitor complex, while rapid kinetic studies have helped to elucidate the mechanism of association [10].

Here, we report dissociation constants for a number of different boronic acid inhibitors of the bacterial protease subtilisin Novo (EC 3.4.21.14). One of these, *N*-dansyl-3-aminobenzeneboronic acid (or *N*-(5-dimethylamino-1-naphthalenesulfonyl)-3-aminobenzeneboronic acid, abbreviated as Dns-BBA) is a fluorescent boronic acid first prepared in [11], for use in cytological experiments as a carbohydrate ligand. We find that the introduction of the dansyl group makes this compound the most potent boronic acid serine protease inhibitor yet found. This inhibitor also exhibits a significant increase in fluorescence intensity upon binding.

We also report binding constants for a variety of other newly studied arylboronic acids. Halogenated benzeneboronic acids are good subtilisin inhibitors, with dissociation constants $<10^{-5}$ M.

2. Materials and methods

Lyophilized subtilisin Novo was a gift of Novo Pharmaceuticals (Wilton CT). Its concentration was determined by use of a second-order rate assay using 4-nitrophenylbutyrate [12]. The concentration of subtilisin solutions after passage on Sephadex G-25 was determined by using the extinction coefficient in [13]. 4-Nitrophenylbutyrate was purchased from Sigma. Dns-BBA was purchased from Aldrich. 4-Tolueneboronic acid was prepared as in [14]. 3-Succinylamidobenzeneboronic acid was prepared as in [15]. 3,5-Bis-(trifluoromethyl)benzeneboronic acid and 2,4-dichlorobenzeneboronic acid were purchased from Alfa/Ventron. 1-Naphthaleneboronic acid was purchased from Bader/Aldrich. *R*-1-Acetamido-2-phenylethaneboronic acid [16] was a gift of Professor D. S. Matteson. Subtilisin kinetics were observed spectrophotometrically using 4-nitrophenylbutyrate as a substrate in 0.1 M ionic strength buffers at 25°C [12]. Substrate hydrolyses were observed in a Schoeffel/McPherson spectrophotometer. Fluorescence studies were done using a Hitachi/Perkin-Elmer MPF-3A spectrofluorimeter. The spectra were observed in 0.1 M ionic strength buffers at 27°C and were corrected for background emission. For enzymatic reactions, buffers used were phosphate (pH 5–8), ammonia (pH 8–9) and bicarbonate (pH 9–10) [17].

3. Results and discussion

3.1. *Effect of structure on inhibitor binding constants*

Table 1 shows dissociation constants for the binding of various arylboronic acids to subtilisin. These data were collected at pH 7.0. Since pK_1 (see below) for various arylboronic acids is usually near 7 [2], it might be expected that the limiting value for these constants is ~1/2 that shown here.

The data in table 1 are consistent with observations showing that electron-withdrawing groups enhance the affinity of boronic acids to subtilisin [2,18]. Such an explanation most reasonably accounts for the very

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Table 1
Boronic acid inhibition constants at pH 7.0

Inhibitor	K_i (M)
N-Dansyl-3-aminobenzeneboronic acid	1.8×10^{-6}
R-1-Acetamido-2-phenylethaneboronic acid	4.0×10^{-6}
3,5-Bis-(Trifluoromethyl)-benzeneboronic acid	5.3×10^{-6}
2,4-Dichlorobenzeneboronic acid	9.4×10^{-6}
1-Naphthaleneboronic acid	4.5×10^{-5}
3-Succinylamidobenzeneboronic acid	2.2×10^{-4}
4-Toueneboronic acid	4.5×10^{-4}

good affinity of the fluorine- and chlorine-substituted boronic acids listed here. The electron-withdrawing halide side chain groups are unlikely to fit into any substrate binding site, yet they clearly do enhance binding affinity. In contrast, the methyl group in 4-toueneboronic acid does not greatly enhance the affinity of this compound to subtilisin, when compared to the parent benzeneboronic acid [2]. However, the sterically similar 4-bromobenzeneboronic acid [2] is a much better inhibitor, presumably because of electronic reasons.

Since these K_i-values are not limiting values, and since pK_1-values have been observed to vary somewhat, it is not appropriate to calculate a Hammett ρ-value from these data. However, the data in table 1, when taken with published values, are consistent with the $\rho = 2.7$ in [18]. This ρ is similar to that shown on ionization of boronic acids, and is presumably due to the similarity between the tetrahedral form of the bound inhibitor (fig.1) [7,9] and the tetrahedral form of the ionized inhibitor [20].

Dns-BBA, with a K_i of 1.8×10^{-6} M at pH 7, clearly deviates from the pattern shown in table 1, since its side chain group would be expected to have an electronic effect similar to that of 3-succinylamidobenzeneboronic acid. The enhanced binding ability of Dns-BBA may be due to either some specific binding effect brought about by the Dns group in the *meta*-position, or it may be due to the effects of increased inhibitor hydrophobicity on K_i. This last suggestion is supported to some degree by the fact that 1-naphthaleneboronic acid has a K_i 4.5-fold better than that of benzeneboronic acid at this pH [2]. Since Dns-BBA has both the naphthalene and benzene rings, its tighter binding might be expected simply on the basis of hydrophobicity alone [21]. However, we think that this is unlikely to be the only explanation for the tight binding shown by Dns-BBA. We are continuing studies on protease inhibition by this reagent.

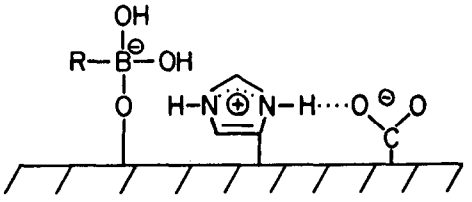

Fig.1. Configuration of boronic acid inhibitors in the active site of serine proteases [7].

The binding constant for the second compound listed in table 1, an analogue of *N*-acetyl-L-phenylalanine, shows that binding constants of substituted benzeneboronic acids are similar to that for a compound that was prepared in order to resemble as closely as possible the transition state in the enzymatic reaction. However, this amino acid analogue (prepared by Matteson and coworkers [16]) has a valuable potential for further elaboration into more specific inhibitors. The binding constant reported here is similar to that found for chymotrypsin [16].

3.2. *pH Dependence of binding*

Fig.2 shows the pH profile for the binding of Dns-BBA to subtilisin. As has been observed for other boronic acids [2], inhibition is dependent on two pK values, one always near 7.2, and the other at various points above this. The first pK is identical to the pK observed in acylation reactions using specific or non-specific substrates. The second pK has been observed to vary with the boronic acid used. Unlike chymo-

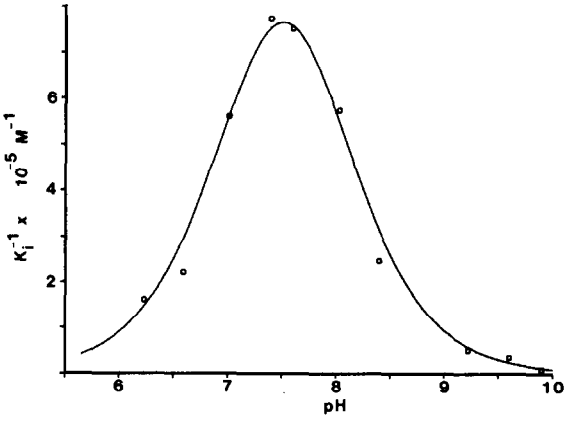

Fig.2. pH profile for the inhibition of subtilisin by Dns-BBA. The theoretical curve corresponds to $pK_1 = 7.2$, $pK_2 = 7.8$, and K_i (lim) = 7×10^{-7} M. Reaction conditions are described in section 2.

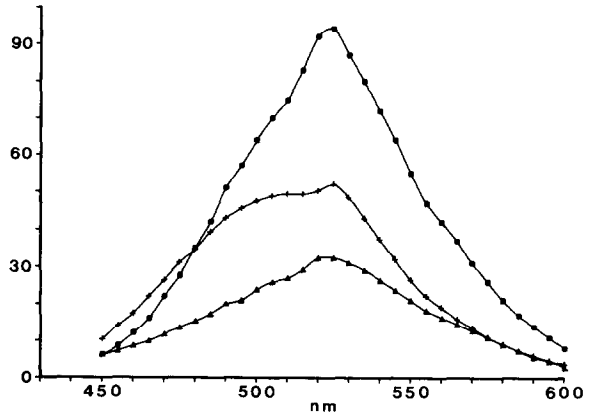

Fig.3. Fluorescence emission of Dns-BBA: In the presence of subtilisin at pH 7.8 (•), in the absence of subtilisin at pH 7.8 (▲), and in the absence of subtilisin at pH 9.2 (+). Subtilisin is 3.5×10^{-5} M and the Dns-BBA is 1.2×10^{-5} M. The ordinate is labeled with arbitrary units proportional to the intensity of fluorescence. Conditions are given in section 2.

trypsin, subtilisin exhibits no pH-dependent variations in binding constants for neutral ligands over pH 4–10 [12,22,23]. The pH profile shown in fig.2 supports the observation [2] that it is the neutral, trigonal boronic acids that bind to the alkaline form of the enzyme active site.

3.3. *Inhibitor fluorescence*

Fig.3 shows that the fluorescence emission of Dns-BBA is markedly enhanced by binding to subtilisin. This enhancement is greater than that seen on ionization of the inhibitor. We thank that this fluorescence enhancement may be useful in studying the mechanism of boronic acid–enzyme interactions. Ultrastructural studies using Dns-BBA may reveal the location of not only certain carbohydrates, but also the location of serine proteases. Since the affinity constants of Dns-BBA to carbohydrates have not been measured, it is unclear which effect is predominant in cytological studies [11].

We attempted to observe the fluorescence emission of 1-naphthaleneboronic acid when bound to subtilisin. However, a high subtilisin concentration is needed to bind a significant proportion of the inhibitor, as binding is weaker than that for Dns-BBA. Since the excitation maximum for 1-naphthaleneboronic acid overlaps significantly with the absorbance maximum of the enzyme, the high enzyme concentration made it difficult to reliably observe inhibitor fluorescence.

We are continuing to study the fluorescence enhancement seen upon binding of Dns-BBA to subtilisin.

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