Proceedings of the National Academy of Sciences
Vol. 68, No. 2, pp. 478–480, February 1971

Inhibition of Serine Proteases by Arylboronic Acids

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Communicated November 30, 1970

ABSTRACT **Arylboronic acids were found to be strong competitive inhibitors of subtilisin and chymotrypsin. The binding constants are strongly pH dependent and give a Hammett-type plot with a slope of −0.885. The pH dependence, the Hammett plot, and nmr model-system studies indicate that inhibition is due to electron-pair donation by the active site histidine to the bound inhibitor.**

The study of chymotrypsin has often been facilitated by the use of nonspecific inhibitors. In addition to serving such direct functions as labeling essential groups of the protein, they are often used in such secondary roles as in the determination of enzymatic reaction rates in relaxation and deacylation studies. Inhibitors can also be a part of rational purification schemes.

An inhibitor that binds strongly but yet is easily removable from the binding site by slight changes in pH can present obvious advantages in such protein studies. Equally advantageous is the availability of a simple chemical moiety which, when attached to an otherwise nonspecific molecule, confers the ability to competitively bind to the desired protein. The boronic acid group, whose use was pioneered by I. V. Berezin, *et al.* (1, 2) is an example of such a moiety. We think that the availability of this group will greatly facilitate the design of compounds that may have other desirable properties, such as a visible chromophore or a spin label.

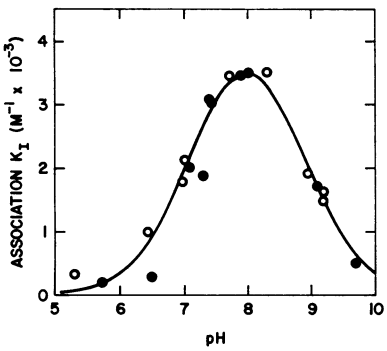


FIG. 1. pH dependence of K_I^{-1} (M^{-1}) for benzeneboronic acid inhibition of subtilisin. Ethyl *N*-acetyl-L-tyrosinate(●), and methyl *N*-acetyl-L-tryptophanate(○), were used as substrates. Buffers below pH 6 are acetate, between pH 6 and 8 are phosphate, and above pH 8 are ammonia, carbonate, and pyrophosphate.

METHODS

The inhibition constants for a series of ring-substituted benzeneboronic acids were determined as a function of pH with chromophoric substrates in buffered solutions. The inhibition constants were then extracted from time-course data (3) using equations given in Webb (4) for competitive inhibitors. Substrates used were ethyl *N*-acetyl-L-tyrosinate (5), and methyl *N*-α-acetyl-L-tryptophanate (6) for chymotrypsin, and these plus phenyl hippurate and *p*-nitrophenyl acetate (7) for subtilisin. Buffers used were acetate, phosphate, ammonia, and carbonate.

Stopped-flow studies were done in a Durrum-Gibson stopped-flow apparatus. In this apparatus, equal amounts of a benzeneboronic acid solution (0–6 × 10^{-3} M) were mixed with a 2.54 × 10^{-5} M proflavin–3.20 × 10^{-5} M α-chymotrypsin solution. The absorbance was measured at 465 nm (ref. 8) 10 msec after mixing. The solvent used was pH 6.75 phosphate buffer, with an ionic strength of 0.2.

The nmr studies were done at 38°C on a Bruker 90-Mhz instrument operating at 28.87 Mhz for the ^{11}B nucleus. The solvent was anhydrous methanol; methyl borate was used as an external standard.

Three times recrystallized α-chymotrypsin was purchased from Worthington Biochemicals, Freehold, N.J. Novo subtilisin was purchased from the Enzyme Development Corp., 64 Wall Street, N.Y.

RESULTS

Enzyme inhibition studies

The inhibition constant data for the various ring-substituted benzeneboronic acids was plotted as $1/K_I$ (in M^{-1}) versus pH. A bell-shaped curve resulted in every case (as in Fig. 1), and could be resolved to a $1/K_I$(lim) and two pK values. The first pK, for every inhibitor, was near 7. This approximates the pK of the enzyme catalytic constant, which results from the imidazole at the active site.

The other pK varied with the inhibitor used; thus, this pK must result from inhibitor ionization. It could not result from an enzyme-binding pK since subtilisin shows no such pK in the binding of neutral substrates (7, 9). With one exception, the pKs observed also approximate those in the literature (10). We then plotted the value of the second pK versus log $1/K_I$(lim) and obtained a least-squares slope of −0.895 with a correlation coefficient of 0.96. This indicates that the inhibitors' affinity towards subtilisin approximates their affinity towards a hydroxyl group*. This is seen in Fig. 2.

* M. P. was an NIH Predoctoral Fellow, 1968–1970.

* Boronic acids probably ionize as *Lewis* acids.

TABLE 1. *Inhibition by boronic acids and benzyl alcohol*

Enzyme	Inhibitor	K_I	pK_1	pK_2	pK*
α-Chymotrypsin	Benzeneboronic acid	1.96×10^{-4} M	7.0	8.42	8.83
α-Chymotrypsin	Benzyl alcohol	1.0×10^{-2} M†	none	9.0†	none
Subtilisin	Benzeneboronic acid	$2.29 + 10^{-4}$ M	7.1	8.92	8.83
Subtilisin	*m*-Nitrobenzeneboronic acid	1.04×10^{-5} M	7.0	7.25	7.23
Subtilisin	*m*-Aminobenzeneboronic acid	1.32×10^{-4} M	7.0	8.66	...
Subtilisin	*p*-Bromobenzeneboronic acid	2.27×10^{-5} M	7.37	8.10	8.66
Subtilisin	Benzyl alcohol (pH 8.4)	1.1×10^{-1} M	...	...	none

* Ref. (10).
† Ref. (16).

To compare the inhibition constants with those of analogous compounds without the boronic acid group, the K_I of benzyl alcohol with subtilisin was determined and a K_I value for benzyl alcohol with α-chymotrypsin was taken from the literature. As can be seen in Table 1, benzyl alcohol binds to these enzymes much more poorly than does benzeneboronic acid at its optimal pH. In addition, benzyl alcohol shows no pH dependence in binding near pH 7 (11).

Finally, we found no time dependence in the apparent K_I values. This was shown first in the millisecond range, using proflavin (8) in a stopped-flow apparatus as an indicator for chymotrypsin–inhibitor binding. The binding was too fast to see and the equilibrium proflavin absorbance appeared immediately after mixing. When an enzyme–inhibitor mixture was repeatedly assayed over a period of minutes, the apparent K_I also remained constant. This was confirmed when it was noted that the time course of inhibited subtilisin reactions, at low substrate concentration, was strictly first-order, as was the time course of chymotrypsin reactions where the inhibitor concentration was high enough to make the expression

$$V = V_m \frac{[S]}{[S] + K_m (1 + [I]/K_I)}$$

become effectively

$$V = V_m[S]/K_m \left(1 + \frac{[I]}{K_I}\right)$$

Again, if binding or debinding of the inhibitor was slow, deviations from first-order kinetics would have been observed. In this respect (unlike its pH dependence), the inhibitor behaves as a normal, substrate-like, binding agent.

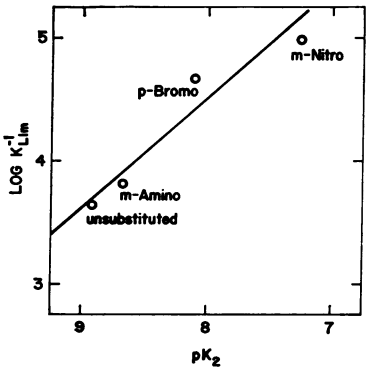


FIG. 2. Hammett-type plot of the substituent effect of the inhibition of subtilisin by benzeneboronic acids. pK_2 is derived from the observed pH dependence of the inhibitions.

Boron-11 model system studies

Boron-11 spectra of model systems were obtained since literature data show that, for boron trifluorides and boric acid, complexation by a variety of ligands produces a change in the ^{11}B chemical shift of +11–13 ppm (12). This change may be associated with the change from trigonal to tetrahedral symmetry on complexation (13).

We observed a similar change in the ^{11}B nmr positions on formation of the diethanolamine complex of benzeneboronic acid, and on addition of an equimolar amount of imidazole to a *m*-nitrobenzeneboronic acid solution (Table 2). In the second case, the position of the ^{11}B nmr peak varied continuously with composition between the chemical shift of the pure boronic acid and that of the equimolar mixture. In the case of the diethanolamine complex, on the other hand, two peaks were obtained, each at a fixed position, when a mixture of complex and free acid ester is present. These results suggest that in the case of the imidazole complex exchange is fast on the nmr time scale. This is consistent with the results obtained above with subtilisin and chymotrypsin.

DISCUSSION AND CONCLUSIONS

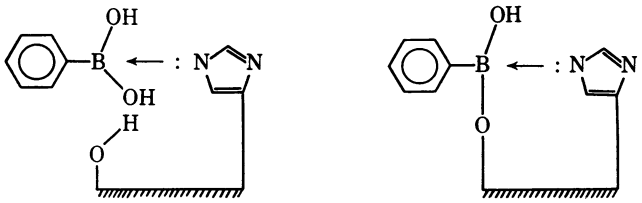
Any reasonable interpretation of the data must explain both the pH dependence and the rapidity of the inhibition. The pH dependence indicates that a neutral inhibitor and a neutral imidazole side chain are involved. Since the inhibitors are competitive with the substrates used, this imidazole is presumably at the active site. Such an imidazole–boronic acid complex is shown in model *A*. This is analogous to the imidazole–boronic acid complexes studied above. However, the existence of the nearby active-site serine suggests model *B* as a possibility. Analogous complexes with an amide (14)

TABLE 2. *nmr Studies of model systems*

Compound	Chemical shift*
Dimethyl benzeneboronate	−9.56 ± 0.03 ppm
Diethanolamine complex of benzeneboronic acid	8.15 ± 0.09 ppm
Dimethyl *m*-nitrobenzeneboronate	−7.55 ± 0.1 ppm
Imidazole complex of dimethyl *m*-nitrobenzeneboronate	11.9 ± 0.1 ppm

* Chemical shift values were taken relative to methyl borate.

or carboxyl (15) group substituting for the imidazole are well known. However, the rate of formation and dissociation of such complexes is quite slow when boric acid and salicylamide derivatives are used. This may be contrasted with the very rapid rates of inhibition observed here. This, taken together with the ^{11}B nmr results, may indicate that imidazole complexation is alone sufficient to explain the inhibition.



A *B*

This work was supported by a grant from the National Institutes of Health. This paper was presented at the 8th International Congress of Biochemistry, Interlaken, Switzerland, September 4, 1970. We also thank Mr. Dan Netzel for performing the nmr analyses.

1. Antonov, V. K., T. V. Ivaniva, I. V. Berezin, and K. Martinek, *Dokl. Akad. Nauk. SSSR (Eng. trans.)*, **183**, 284 (1968).
2. Antonov, V. K., T. V. Ivaniva, I. V. Berezin, and K. Martinek, FEBS *Lett.*, **7**, 23 (1970).
3. Bender, M. L., G. E. Clement, C. R. Gunter, and F. J. Kézdy, *J. Amer. Chem. Soc.*, **86**, 3697 (1964).
4. Webb, J. L., *Enzymes and Metabolic Inhibitors* (Academic Press, New York, 1963), vol. I, pp. 150–151.
5. Schwert, G. W., and Y. Takenaka, *Biochim. Biophys. Acta*, **16**, 570 (1955).
6. Zerner, B., and M. L. Bender, *J. Amer. Chem. Soc.*, **86**, 3674 (1964).
7. Polgar, L., and M. L. Bender, *Biochemistry*, **6**, 610 (1967).
8. Glazer, A. N., *Proc. Nat. Acad. Sci. USA*, **54**, 171 (1965).
9. Glazer, A. N., *J. Biol. Chem.*, **242**, 433 (1967).
10. Juillard, J., and N. Gueguen, *C. R. Acad. Sci. Paris, C*, **264**, 259 (1967).
11. Valenzuela, P., and M. L. Bender, *Biochemistry*, **9**, 2440 (1970).
12. Schaeffer, R., in *Progress in Boron Chemistry*, ed. H. Steinberg, A. L. McCloskey (MacMillan, New York, 1964), vol. I, pp. 453–459.
13. Niedenzu, K., and J. W. Dawson, *Boron-Nitrogen Compounds* (Academic Press, New York, 1965), pp. 156–158.
14. Tanner, D. W., and T. C. Bruice, *J. Amer. Chem. Soc.*, **89**, 6954 (1967).
15. H. Schäfer, *Z. Anorg. Allg. Chem.*, **250**, 82 (1942).
16. Bender, M. L., and F. C. Wedler Jr., *J. Amer. Chem. Soc.*, **91**, 3894 (1969).