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Contents (*Continued from outside back cover*)

Contents

Vol. 68, No. 2 February 1971

Physical Sciences

Biological Sciences

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.

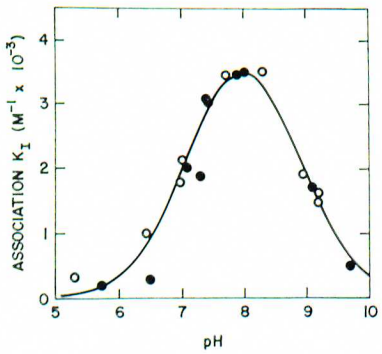

FIG. 1. pH dependence of K_I^{-1} (M^{-1}) for benzeneboronic acid inhibition of subtilisin. Ethyl *N*-acetyl-L-tyrosinate(●), and methyl *N*-acetyl-L-tryptophanate(○), were used as substrates. Buffers below pH 6 are acetate, between pH 6 and 8 are phosphate, and above pH 8 are ammonia, carbonate, and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

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