n-ALKYLBORONIC ACIDS AS BIFUNCTIONAL REVERSIBLE INHIBITORS OF α-CHYMOTRYPSIN

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1. Introduction

Study of the interaction of enzymes with polyfunctional reversible inhibitors may yield valuable information on the spatial arrangement of the active site. Earlier [1] we proposed the use of alkylboronic acids as such inhibitors in studies of hydrolytic reactions catalysed by α-chymotrypsin. These compounds interact with the enzyme by attachment of the boric acid residue to the catalytically active imidazole grouping, while the alkyl chain, if long enough, penetrates into the hydrophobic loci of the active site. The present paper describes the inhibitory action of a homologous series of n-alkylboronic acids $H(CH_2)_nB(OH)_2$, where n = 2–8, in the α-chymotrypsin-catalysed hydrolysis of some synthetic substrates. The inhibitory properties depended on the length of the alkyl chain. The relationship observed led to some new suggestions concerning the structure of the active site of the enzyme.

2. Experimental

The n-alkylboronic acids were prepared either according to a modified procedure of Snyder et al. [2] or to the method of McCusker et al. [3]. *p*-Nitrophenyl trimethylacetate (NPTMA) [4], *N*-acetyl-L-tyrosine ethyl ester (ATEE) [5], *N*-(3-carboxypropionyl)-L-phenylalanine *p*-nitroanilide (CPPA) [6] served as substartes. Crystalline α-chymotrypsin was obtained from the Leningrad meat packing plant [7].

The enzymatic hydrolysis kinetics of ATEE were determined by means of a Radiometer TTT-1c, pH-stat at $[S]_o \gg [E]_o$, 30°C, in 0.1 M NaCl containing 10% (v/v) CH_3OH. Under these conditions, the rate of the initial steady-state enzymatic reaction obeys the Michaelis-Menten equation, $v = V_{max}[S]_o/(K_{m(app)} + [S]_o)$, where $K_{m(app)} \geqslant 10^{-2}$ M. The inhibition of this reaction by n-alkylboronic acids was investigated at $[S]_o = 6.1 \times 10^{-4}$ M with $[E]_o$ being varied from 10^{-6} to 10^{-8} M. The course of the enzymatic hydrolysis of CPPA and NPTMA under the above conditions was followed spectrophotometrically on a Hitachi EPS-3 instrument (0.01–0.02 M tris-HCl buffer). The pres-teady-state kinetics of the α-chymotryptic hydrolysis of NPTMA were investigated at $[E]_o = [S]_o = 4 \times 10^{-5}$ M (as described elsewhere [8]) while the steady-state enzymatic hydrolysis of CPPA was carrier out in a spectrophotometric cell at $[S]_o \gg [E]_o = 10^{-6}$ to 10^{-7} M [6]. It was found that: $K_{m(app)} = (1.5 \pm 0.3) \times 10^{-3}$ M.

3. Results and discussion

At $K_{m(app)} \gg [S]_o$, the hydrolysis of amides and esters catalysed by α-chymotrypsin can be represented as follows (1) [9]:

$$E + S \xrightarrow{k_2/K_s} E + P_1 + P_2 \qquad (1),$$

where E denotes the enzyme, S is the substrate, P_1 and P_2 are the reaction products. A linear relationship between the inhibitory effect of n-hexylboronic acid and its concentration was shown. This agrees with the reaction schemes (1) and (2):

$$E + I \rightleftharpoons EI \qquad (2),$$

where I is the inhibitor. When $[I] \gg [E]_0$ we have

$$v/v_i = 1 + K_i[I] \qquad (3),$$

where v_i and v are the steady-state rates of the enzymatic process measured in the presence and absence of inhibitor, respectively. Hence the association constant K_i can be determined from the slope of the plot v/v_i vs. [I]. The K_i values for the alkylboronic acids investigated are given at varying pH values in fig. 1.

It follows from fig. 1 that reversible binding of n-alkylboronic acids sharply decreases at pH < 7. This cannot be explained by changes in ionic state of the inhibitor, since its pK_a exceeds 9 (H_3BO_3, $pK_a = 9.2$; $C_2H_5B(OH)_2$, $pK_a = 10.2$), but can be accounted for by changes in the ionic state of an ionogenic group in the enzyme with a $pK_a \sim 7$. Fig. 1 shows that the changes in K_i obey Eq. (4) if it is assumed that the inhibitor interacts with the basic form of the enzyme (E), the pK_a of the ionogenic group being 6.6–6.8.

$$EH^+ \overset{K_a}{\rightleftharpoons} E \overset{K_{i(\lim)}}{\rightleftharpoons} EI \qquad (4)$$
$$K_i = K_{i(\lim)}(1 + [H^3]/K_a)$$

Earlier [7, 10] is was shown that the ionogenic group involved in boric acid inhibition of α-chymotrypsin was the catalytically active imidazole of the histidine-57 residue. Using two-component inhibition [11], we found that n-alkylboronic acids compete with boric acid for the same binding site. Hence it was reasonable to suppose that in alkylboronic acid inhibition, the imidazole moiety of histidine-57 also interacts with alkylboronic acids.

The dependence of $K_{i(\lim)}$ (the limiting value of K_i at pH > 7) on the alkyl chain length of the inhibitor is shown in fig. 2; n-Alkylboronic acids with relatively short chains ($n \leqslant 3$) have almost the same inhibitory properties as H_3BO_3. However, starting with butylboronic acid (n = 3), $\log K_{i(\lim)}$ increases linearly with increase in n reaching a mximum at n = 6. From this we conclude that alkylboronic acids with adequate chain lengths also interact with the hydrophobic active site in chymotrypsin. Results from a two component inhibition study of n-hexylboronic acid and competitive phenol also support this view.

The increment of the free energy of binding ($\Delta\Delta F_{i(CH_2)}$) due to each methylene group in alkylboronic acids for $3 < n \leqslant 6$ is −700(± 100) cal/mole. This agrees with the value of $\Delta\Delta F_{i(CH_2)}$ found earlier [12] for the sorption of aliphatic alcohols by the active site of chymotrypsin. Hence, it follows that for

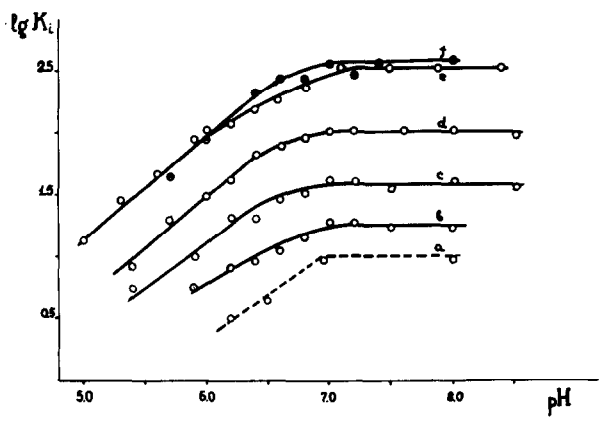

Fig. 1. pH-Dependence of the apparent $RB(OH)_2$ inhibition constant in the enzymatic hydrolysis of ATEE.
R: a = C_2H_5, b = n-C_3H_7, c = n-C_4H_9, d = n-C_5H_{11}, e = n-C_6H_{13}, f = n-C_7H_{15}.

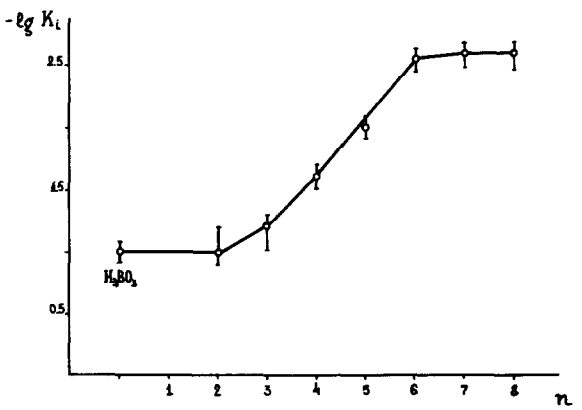

Fig. 2. Plot of $K_{i(\lim)}$ versus n in $H(CH_2)_nB(OH)_2$.

$n > 3$, n-alkylboronic acids become bifunctional inhibitors, interacting simultaneously with the catalytically active imidazole residue and the hydrophobic active site. The constant value of $K_{i(lim)}$ for $n > 6$ indicates the limited size of the hydrophobic site.

It is interesting that chymotrypsin also exhibits maximal kinetic specificity when n = 6 for substrates such as $H(CH_2)_nCOOAr$ [13] or $H(CH_2)_{n-1}CH(NHAc)COOCH_3$ [14]. The increments of the free energy of activation $\Delta\Delta F^*_{(CH_2)}$ of the rate limiting chemical stage of the enzymatic process for these substances are −700 and −650 cal/mole, respectively. Since the state of the alkylboronic acids bound to the enzyme simulates the that of substrate in the acyl-enzyme ("bidental binding"), the evidence obtained here confirms the assumption that hydrophobic interaction between the side chain of the substrate molecule and the corresponding enzyme site occurs at this stage of the reaction.

Earlier [14], this conclusion was based only on indirect data obtained from kinetic measurements of the free energy of activation ΔF^*_{cat} of rate limiting chemical stage of enzymatic hydrolysis. Detailed elucidation of the mechanism of formation of the active center (i.e. whether it is a conformationally rigid active center in the free enzyme or whether the complementarity is induced by the substrate) requires additional study in the course of which bifunctional reversible inhibitors could serve as useful tools.

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