BINDING–CATALYSIS RELATIONSHIP IN α-CHYMOTRYPSIN ACTION AS REVEALED FROM REVERSIBLE INHIBITION STUDY OF PHENYLALKYLBORONIC ACIDS

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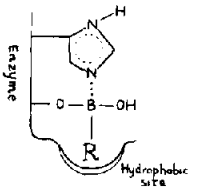
1. Introduction

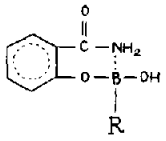
Studies of the reversible inhibition of α-chymotrypsin (CT) by n-alkylboronic acids have supplied important information on the topography of the enzyme's active centre [1, 2]. The hydrocarbon moiety of these bifunctional inhibitors interacts with the hydrophobic region of the CT active centre, whereas the borate grouping forms a complex with its catalytic functional groups. The following facts point to participation of the catalytically active histidine-57 residue in the complex formation: (1) both H_3BO_3 [3] as well as the alkylboronic acids [1, 2], and also phenylboronic [4] and phenylethylboronic [5] acids are reversible, pH-dependent inhibitors of CT, the pK_a of the protein group controlling the degree of inhibition being 6.5–7; (2) H_3BO_3 competes with Cu^{2+} ions for binding by the CT active centre [6]; also it has been unequivocally demonstrated that Cu^{2+} ions interact with the active-site imidazole [7, 8]; (3) H_3BO_3 reversibly inhibits alkylation of the catalytically active imidazole by 1,4-dibromo-2-phenylacetoin [9, 10]. In the light of present knowledge of borate complex chemistry (see e.g. [11]) and of the structure and mode of action of the CT active centre, these facts have led to the proposal that the OH group of the serine-195 residue also participates in the enzyme–borate complex, forming an ester bond (scheme 1) [12]. Such an inhibition mechanism has been also discussed by other investigators [4, 5].

It was of interest to elucidate the inhibiting capacity of another series of bifunctional reversible CT inhibitors, namely phenylalkylboronic acids, which are closer analogues than the alkylboronic acids of the specific substrates of the enzyme. We have studied the relation between structure and inhibiting capacity of phenylalkylboronic aicds, $RB(OH)_2$, where $R = C_6H_5(CH_2)_n-$ with n varying from 0 to 4*. In the quantitative treatment of the results it must be borne in mind that, besides the hydrophobic interaction with the enzyme, the substituent R may also contribute to the over-all action of these bifunctional inhibitors by the influence it can exert on the "complexing" properties of the borate group (in other words account must be taken of the R substituent's steric and inductive effects). To this end we investigated the complexing of substituted boronic acids with salicylamide. Boronic acid–salicylamide complexing was first studied by Tanner

* While this paper was in preparation, phenylboronic [4] and phenylethylboronic [5] acids were found to be strong competitive inhibitors of CT.



Scheme 1.



Scheme 2.

and Bruice [11]. This process, which involves a low-molecular ligand (see scheme 2), may be regarded as stimulating the CT–substituted boronic acids complexing reaction. In the model process, however, there is no additional hydrophobic interaction of R with the protein.

2. Materials and methods

CT was supplied by the Leningrad meat-packing plant [6]. The characteristics of salicylamide were the same as in [11]. The n-alkylboronic acids have been described [2]. Phenylboronic acid was a commercial preparation (Schuchardt), recrystallized fron water. The phenylalkylboronic acids were synthesized according to [2]: phenylethylboronic acid, m.p. 83–84°; phenylpropylboronic acid, m.p. 78–80°; and phenylbutylboronic acid, m.p. 81–82°. The purity of the phenylalkylboronic acids was assessed from their elemental analyses.

The K_i inhibition constants were determined as described [1, 2], with the aid of a Radiometer TTT 1c pH-stat using *N*-acetyl-L-tyrosine ethyl ester as substrate.

The boronic acid–salicylamide complexing reactions were followed spectrophotometrically by recording the disappearance of the salicylamide band at 333 nm [11]. The measurements were performec with a Hitachi Perkin-Elmer 124 instrument.

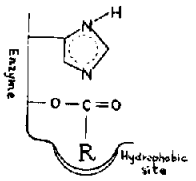
3. Results and discussion

The hydrocarbon grouping of the substituted boronic acid molecule has relatively little effect on the complexing of the borate group with salicylamide. Although the apparent association constant, K_{app}, does show a certain dependence on the presence and the structure of a hydrocarbon group (see table 1), the observed changes do not exceed the limits of the ordinary inductive and steric effects inherent in electrophilic–nucleophilic interactions [13]. Quite different is the complex formation of the boronic acids with the CT active centre. The values of the inhibition constants, K_i (see table 1), are in this case greatly dependent upon the hydrophobic capacity (log P) of the R substituent in the inhibitor molecule, $RB(OH)_2$. For instance, as can be seen from table 1, the interaction of salicylamide with both H_3BO_3 and phenylethylboronic acid is characterized by approximately equal values of the

Table 1.
Complexing of boronic acids, $RB(OH)_2$, with α-chymotrypsin active centre (K_i) and with salicylamide (K_{app})

R	K_i [a,b] 1/mole	K_{app} [c,d] 1/mole
HO-	10 [e]	33 ± 10
Phenyl	780	140 ± 20
Phenylethyl	10,950	28 ± 8
Phenylpropyl	5,200	33 ± 10
Phenylbutyl	3,300	–
n-Butyl	40 [e]	15.5 ± 3.5
n-Amyl	105 [e]	12.5 ± 2.5
n-Hexyl	370 [e]	10.5 ± 1.5

[a] pH 8.0 (pH-stat), 25°, 0.1 M NaCl, 10 vol.% of CH_3OH.
[b] Accuracy of measurement of K_i values is about 10%.
[c] pH 8.0 (1/15 M phosphate buffer), 20°, 0.02 M NaCl, 10 vol % of CH_3OH.
[d] Mean and standard deviation of not less than 5 measurements.
[e] Taken from [1, 2].



Scheme 3

apparent association constant, K_{app}. However, the CT active centre reversibly binds phenylethylboronic acid a thousand times more effectively than H_3BO_3. The increment of free energy, favouring CT–inhibitor binding, is equal to the free energy of transfer of the $C_6H_5(CH_2)_2-$ group from water to an organic solvent (about −4.3 kcal/mole for the system n-octanol–water [14]).

The dependence of the inhibiting capacity of phenylalkylboronic acids, $C_6H_5(CH_2)_nB(OH)_2$, on the length of the side chain (n) has a maximum at the compound whose aralkyl grouping is nearest in size to the phenylalanine derivative ($n = 2$) which is the specific substrate of the enzyme.

If, assuming that in the acylenzyme the substrate is also bound to the enzyme at two other points (scheme 3) the bifunctional inhibitor–CT active centre complex (scheme 1) may be regarded as simulating the acylenzyme*, with hydrophobic interaction between the substituent and the protein apparently being common to both systems. In this connection it is of interest to analyse the relationship between the free energy of the enzyme–inhibitor binding and of the activation of acylenzyme deacylation. To this end, in fig. 1 along with our results (K_i) we have given also data [15] on the deacylation rate (k_3) of $C_6H_5(CH_2)_nCO$-chymotrypsins. It is evident that the binding efficiency ($\log K_i$) changes parallely with changes in the catalytic

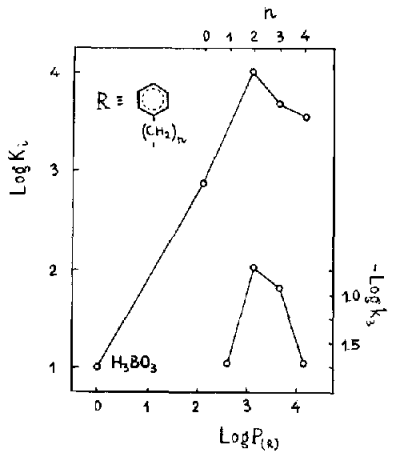


Fig. 1. The effect of the substituent R hydrophobicity (log P) upon the binding constant (K_i) in the α-chymotrypsin–$RB(OH)_2$ complexing reaction and upon the deacylation rate constants of acylchymotrypsins, RCO-enz. Experimental conditions for measuring the inhibition constants, K_i, are given in the table. The k_3 values are from [15]. The P values were taken from the data in [14].

efficiency ($\log k_3$), both systems exhibiting maximum efficiency for same size of side group, $n = 2$. These data supply direct proof of the earlier expressed suggestion that the thermodynamically favourable free energy of the enzyme–substrate binding causes a decrease in the free energy of activation of the chemical reaction, i.e. these data confirm the postulate advanced by Knowles: "Better binding – better catalysis!" [16, 17].

* We do not consider it necessary to regard a bifunctional inhibitor as a transition state analog, as was assumed in [5]. If the hydrophobic binding of the R grouping with the protein favors catalytic reaction, the gain in free energy of activation should be the same independently of whether the binding occurs in the ground state of the reaction (in the acylenzyme) and is retained in the transition state or has its source only at the transition state [18].

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