KINETIC ASPECTS OF THE IRREVERSIBLE INHIBITION OF TRYPSIN AND RELATED ENZYMES BY *p*-[*m*(*m*-FLUOROSULFONYLPHENYLUREIDO)PHENOXYETHOXY] BENZAMIDINE

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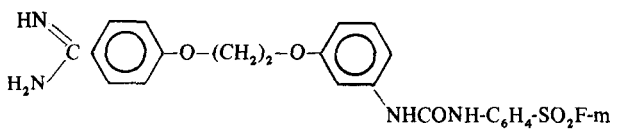
1. Introduction

In recent years Baker and Erickson have achieved considerable success in producing potent active-site-directed irreversible inhibtors of trypsin [1, 2]. These compounds are derivatives of (phenoxyalkoxy)benzamidine and they undergo covalent bonding to the enzyme with the help of a terminal sulfonylfluoride group. One of the strongest inhibitors was found in *p*-[*m*(*m*-fluorosulfonylphenylureido)phenoxyethoxy] benzamidine [2]:



This communication is a more detailed account of the kinetic parameters of the interaction of the inhibitor with trypsin and, in addition, describes the effect of the compound on pancreatic kallikrein, thrombin, and plasmin.

2. Experimental procedure

2.1. *Materials*

p-[*m*(*m*-Fluorosulfonylphenylureido)phenoxyethoxy] benzamidine benzene sulfonate (FPPB) was a product of Cyclo Chemical. Bovine trypsin (2 X crystallized; salt free), *N*α-benzoyl-L-arginine ethyl ester-HCl (BAEE) and *p*-tosyl-L-arginine methyl ester-HCl (TAME) were obtained from Schwarz-Mann. Lyophilized hog pancreatic kallikrein was kindly supplied by Farbenfabriken Bayer AG. The material was free of trypsin and contained 125 KU/mg (KU = inhibitor units according to Frey et al. [3]). Bovine thrombin (topical) was purchased from Parke, Davis and Co. Human plasmin in 50% glycerol solution was obtained from *AB* KABI.

2.2. *Methods*

The enzymes were incubated for various periods of time with the inhibitor and afterwards the amount of residual enzymatic activity was determined in rate assays. The degree of dilution of the inhibition mixtures following incubation and the concentration of the substrates in the assays were chosen such that they eliminated any continuing reversible or irreversible blocking effect of FPPB. The composition of the inhibition mixtures was as follows: for trypsin, 0.4 ml of 0.05 M Tris-HCl buffer pH 8.1 contained 0.5 – 5.7 μg of the enzyme, 0.75 – 12 X 10^{-6} M FPPB, 0.02 M $CaCl_2$ and 2.5% v/v dimethylsulfoxide (DMSO). The larger amounts of trypsin were employed with the higher inhibitor concentrations to make possible a greater dilution before the assay step and still to be able to measure trypsin accurately. For kallikrein, 0.8 ml of 0.01 M Tris-HCl buffer pH 8.1 included 1 KU of the enzyme, 2 – 8 X 10^{-6} M FPPB and 2.5% v/v DMSO. For thrombin, 200 NIH units of the enzyme and 1 – 6.4 X 10^{-4} M FPPB were incorporated into 0.5 ml of 0.02 M phosphate buffer pH 8.0 containing 4% v/v DMSO. For plasmin, 0.6 ml of 0.05 M Tris-HCl buffer pH 8.1 contained 2 units (casein units according to Sgouris [4]) of the enzyme, 3.3 – 13 X 10^{-5} M

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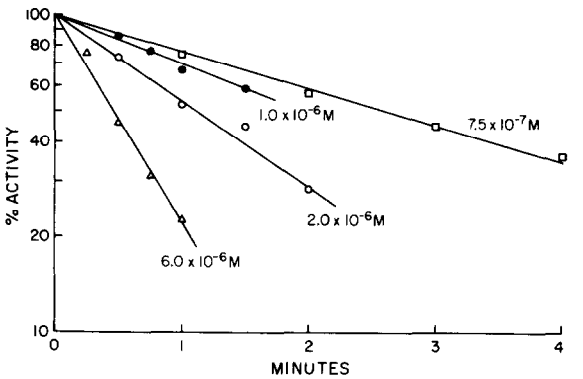

Fig.1. Loss of trypsin activity upon incubation of the enzyme with various concentrations of FPPB at 37° and pH 8.1.

FPPB, 3.39% v/v DMSO and 17% v/v glycerol. BAEE was employed as substrate to measure the activities of trypsin, plasmin, and kallikrein. With the first two enzymes Schwert and Takenaka's assay method [5] and a BAEE concentration of 1×10^{-3} M were chosen, while with kallikrein a modified Hestrin method [6] and a substrate concentration of 1×10^{-2} M were used. Thrombin activity was determined according to the procedure of Siegelman et al. [7] by measuring the amount of methanol released during the hydrolysis of TAME.

3. Results and discussion

Fig.1 shows that in the presence of an excess of FPPB the inhibition of trypsin followed apparent first order kinetics. From such graphs the pseudo first order rate constants were determined for the inhibitor concentrations chosen, and the values of several experiments were averaged. A double reciprocal plot of the mean pseudo first order constants thus obtained against inhibitor concentration resulted in a straight line which intersected the y-axis above the point of origin (fig.2) and allowed the graphic determination of the limiting rate constant of inactivation at high inhibitor concentrations, i.e., of k_2 [8]. Occurrence of a positive intercept on the ordinate proved the formation of a reversible enzyme–inhibitor complex before the enzyme was irreversibly inhibited, and the reaction sequence can therefore be written as

Table 1
Kinetic parameters for the inhibition of trypsin, pancreatic kallikrein, thrombin and plasmin by *p*-[*m*(*m*-fluorosulfonylphenylureido)phenoxyethoxy] benzamidine (FPPB).

Enzyme	k_2 (min^{-1})	$\frac{t}{2}$ lim *	K_i (M)
Trypsin	3.70	11 sec	8.9×10^{-6}
Kallikrein	0.0425	16 min	4.3×10^{-6}
Thrombin	0.0122	57 min	7.5×10^{-4}
Plasmin	0.0014	478 min	2.5×10^{-5}

* Half-life of the enzyme at the limiting rate of inactivation k_2.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_2} E \cdot I'$$

As (I) was chosen considerably greater than (E) it was possible to calculate the inhibition constant K_i from the formula

$$\frac{1}{k_{app}} = \frac{1}{k_2} + \frac{K_i}{k_2} \times \frac{1}{(I)} \qquad [8]$$

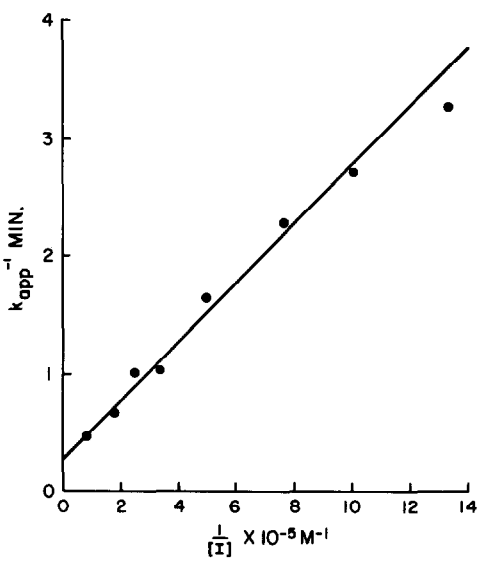

Fig.2. Plots of $1/k_{app}$ against 1/(I) for the inactivation of trypsin by FPPB. The limiting rate of inactivation determined from the positive intercept on the ordinate is identical with k_2.

Plots of $1/k_{app}$ against 1/(I) were constructed also for pancreatic kallikrein, thrombin and plasmin and, as with trypsin, they showed a positive intercept on the ordinate. The kinetic data for all the enzymes are summarized in table 1. It is evident that FPPB is indeed a powerful inhibitor of trypsin. The k_2 value of 3.70 min^{-1} (pH 8.1, 37°) compares favorably with a value of 0.16 min^{-1} as reported for TLCK with β-trypsin (pH 7.0, 25°) [9]. Interestingly, pancreatic kallikrein was also readily inhibited by FPPB though TLCK is without influence on this enzyme [10]. The rate of inactivation of thrombin was less than one third that of kallikrein while plasmin was still considerably less susceptible to inhibition. It should be pointed out that there was no correlation between K_i and k_2 values for different enzymes. Thus, the inhibitor had a much greater affinity for plasmin than for thrombin yet despite this, k_2 was about 9 times greater with thrombin than with plasmin.

Acknowledgement

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