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ON THE IRREVERSIBLE INHIBITION OF α-CHYMOTRYPSIN, TRYPSIN, PANCREATIC KALLIKREIN*, THROMBIN AND ELASTASE BY *N*-(β-PYRIDYLMETHYL)-3,4-DICHLOROPHENOXYACETAMIDE-*p*-FLUOROSULFONYLACETANILIDE BROMIDE

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SUMMARY

1. Kinetic parameters and specificity characteristics have been determined for *N*-(β-pyridylmethyl)-3,4-dichlorophenoxyacetamide-*p*-fluorosulfonylacetanilide bromide (PDFA), a member of a new class of active-site-directed irreversible protease inhibitors where covalent bonding to the enzymes occurs outside the limits of the active site proper. The results establish PDFA as one of the most potent inhibitors of α-chymotrypsin (EC 3.4.4.5) with a second-order rate constant of inactivation of $5.41 \cdot 10^4$ $M^{-1} \cdot min^{-1}$ at pH 7.0 and 37 °C.

2. PDFA is also a weak irreversible inhibitor of trypsin (EC 3.4.4.4), pancreatic kallikrein (EC 3.4.4.21), elastase (EC 3.4.4.7) and thrombin (EC 3.4.4.13), in this approximate descending order of effectiveness. The second-order rate constant of inhibition with trypsin (at pH 8.1) is more than 200 times lower than with α-chymotrypsin (at pH 7.0).

INTRODUCTION

In recent years, Baker and his co-workers[1] have developed large numbers of irreversible chymotrypsin (EC 3.4.4.5) inhibitors of a novel type. Compared with other synthetic inhibitors, these compounds possess a greater bulk, and a larger distance separates their active-site-directed moiety from the moiety binding covalently to the enzyme, *i.e.* the reactive $-SO_2F$ group. Because of their greater size the inhibitors are thought to extend beyond the confines of the active site and thus may be useful as tools for exploring enzyme topography beyond the limits of the active center. As the inhibitors produced by Baker and his group have been characterized only by their approximate inhibitory strength we felt it important to obtain additional kinetic data before proceeding to further applications of the compounds.

Abbreviation: PDFA, *N*-(β-pyridylmethyl)-3,4-dichlorophenoxyacetamide-*p*-fluorosulfonylacetanilide bromide.

* Kallikrein is a registered trademark assigned to Farbenfabriken Bayer AG, Leverkusen, Federal Republic of Germany.

In this communication we therefore present results on the activity and specificity of *N*-(β-pyridylmethyl)-3,4-dichlorophenoxyacetamide-*p*-fluorosulfonylacetanilide bromide (PDFA), the strongest chymotrypsin inhibitor in the series of Baker and Hurlbutt[2].

EXPERIMENTAL PROCEDURES

Materials

Bovine α-chymotrypsin (3 times crystallized, salt-free) and bovine trypsin (EC 3.4.4.4) (2 times crystallized, salt-free) were obtained from Schwarz-Mann. Lyophilized hog pancreatic kallikrein (EC 3.4.4.21) (125 Frey units/mg) was supplied by Farbenfabriken Bayer AG. Bovine thrombin (EC 3.4.4.13) (topical) was purchased from Parke, Davis and Co. Elastase (EC 3.4.4.7) (hog pancreas; lyophilized; 76 Sachar units/mg) was from Worthington.

PDFA and indole-3-pyruvic acid were from Cyclo Chemical. *N*-Acetyl-L-tyrosine ethyl ester, N^{α}-benzoyl-L-arginine ethyl ester HCl, and *p*-tosyl-L-arginine methyl ester HCl were purchased from Schwarz-Mann. Elastin impregnated with orcein was obtained from Worthington.

Methods

To determine irreversible inhibition by PDFA the enzymes were incubated with a large excess of inhibitor at 37 °C for varying times, and subsequently the residual enzyme activity was measured by rate assays. The composition of the inhibition mixtures and the second-stage assay methods for four of the five enzymes studied are given in Table I. With elastase it was not possible to adjust enzyme and

TABLE I

COMPOSITION OF ENZYME INCUBATION MIXTURES FOR THE IRREVERSIBLE INHIBITION BY PDFA AND TYPES OF ASSAYS USED IN THE MEASUREMENT OF RESIDUAL ENZYME ACTIVITIES

ATEE, *N*-acetyl-L-tyrosine ethyl ester; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; TAME, *p*-tosyl-L-arginine methyl ester.

Enzyme	*Composition of inactivation mixtures*			*Assay (substrate; method)*
	Buffer	*PDFA concn (M)*	*Enzyme concn* (M)*	
α-Chymotrypsin	0.025 M imidazole–HCl, pH 7.0, 0.05 M $CaCl_2$	$5 \cdot 10^{-6}$ to $30 \cdot 10^{-6}$	$4.16 \cdot 10^{-7}$ to $25 \cdot 10^{-7}$	ATEE; Hestrin[3]
Trypsin	0.05 M Tris–HCl, pH 8.1, 0.02 M $CaCl_2$	$0.83 \cdot 10^{-4}$ to $7.5 \cdot 10^{-4}$	$1.5 \cdot 10^{-7}$ to $6.8 \cdot 10^{-7}$	BAEE; Schwert and Takenaka[4]
Kallikrein	0.05 M Tris–HCl, pH 8.1	$1 \cdot 10^{-4}$ to $5 \cdot 10^{-4}$	$1.25 \cdot 10^{-5}$ to $6.25 \cdot 10^{-5}$ (750–3750 Frey units/ml)	BAEE; Hestrin[3]
Thrombin	0.02 M phosphate, pH 8.1, 0.002 M NaCl	$2 \cdot 10^{-4}$ to $8 \cdot 10^{-4}$	$5 \cdot 10^{-6}$ M (400 N.I.H. units/ml)	TAME; Siegelman *et al.*[5]

* For chymotrypsin and trypsin the concentrations are based on the assumption of 100% purity of the commercial enzyme preparations. The values therefore represent the theoretically possible upper limits. For pancreatic kallikrein and for thrombin the calculations are based on reported molecular weights and the specific activity of the most highly purified preparations (refs 6 and 7, respectively).

inhibitor concentrations to levels that would permit irreversible inhibition to occur and still allow enough leeway to reduce PDFA concentrations by dilution to non-interfering levels before proceeding to the assay step. Consequently, the kinetic data for elastase reflect the concurrent effects of irreversible as well as reversible inhibition by PDFA. The assay method was that of Sachar *et al.*[8]. The reaction mixtures of 1.5 ml of 0.07 M Tris–HCl buffer (pH 8.8) included 45.6 units of elastase and 10 mg of orcein–elastin. Incubation was carried out for 20 min at 37 °C.

Kinetic considerations

The interaction of enzymes with PDFA can be considered to follow the sequences

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightarrow}} E \cdot I \overset{k_2}{\rightleftharpoons} E \cdot I' + P \overset{k_3}{\rightarrow} E + I' \qquad (1)$$

In this equation $E \cdot I$ represents the initial reversible enzyme–inhibitor complex which at the rate k_2 is converted into the irreversibly inhibited enzyme derivative $E \cdot I'$. Since reactivation of the enzymes studied was very small k_3 could be neglected, and for $[I] \gg [E]$ use could be made of an expression previously developed by Kitz and Wilson[9]

$$k_{app} = \frac{k_2}{K_i + [I]} \times [I] \qquad (2)$$

Here, k_{app} denotes the pseudo-first-order rate of inactivation, and K_i is the dissociation constant of the reversible enzyme–inhibitor complex. From this equation it is evident that in the case of $[I] \ll K_i$ the ratio of $k_{app}/[I]$ will remain constant and will be identical with the second-order rate constant of inactivation. It can also be shown that in the range of the K_i concentration, on the other hand, $k_{app}/[I]$ will become progressively smaller, and plots of $1/k_{app}$ against $1/[I]$ allow us to determine k_2 as the reciprocal of the intercept of the curve on the y-axis. Knowing k_2 it is then possible to solve Eqn 2 for K_i.

RESULTS

In the presence of an excess of PDFA irreversible inhibition of α-chymotrypsin, trypsin, kallikrein and thrombin followed pseudo-first-order kinetics, and, as expected, α-chymotrypsin was inactivated much faster than any of the other enzymes. The apparent first-order rate constants of inhibition are listed in Table II as are the corresponding values of $k_{app}/[I]$. It is evident that for α-chymotrypsin and trypsin the latter ratios are rather constant over the range of PDFA levels employed. Consequently, plots of $1/k_{app}$ against $1/[I]$ result in straight lines that pass through the origin (Figs 1a and 1b), and $k_{app}/[I]$ can be taken as the second-order rate constant of inactivation.

In the case of kallikrein and thrombin, all values of $[I]$ are in the K_i range, and plots of $1/k_{app}$ against $1/[I]$ intersect the ordinate above the point of origin (Figs 1c and 1d) enabling us to obtain k_2 and then to calculate the K_i values.

The kinetic data presented for α-chymotrypsin so far do not provide any evidence for involvement of the active site in the interaction with PDFA. Proof for such a participation, however, comes from experiments in which indole-3-pyruvic

TABLE II

KINETIC PARAMETERS FOR THE IRREVERSIBLE INHIBITION OF α-CHYMOTRYPSIN, TRYPSIN, PANCREATIC KALLIKREIN, AND THROMBIN BY PDFA AT 37 °C

Enzyme	*Inhibitor concentration* ($M \times 10^4$)	k_{app} $min^{-1} \times 10^3$	$k_{app}/[I]$ $(M^{-1} \cdot min^{-1})$	k_2 $(min^{-1} \times 10^3)$	K_i (M)
α-Chymotrypsin (pH 7.0)	0.05	309	$618 \cdot 10^2$		
	0.06	327	$545 \cdot 10^2$		
	0.07	369	$527 \cdot 10^2$		
	0.12	673	$561 \cdot 10^2$		
	0.30	1613	$538 \cdot 10^2$		
Trypsin (pH 8.1)	0.83	18.9	228		
	1.25	26.7	214		
	2.50	54.1	216		
	7.50	192.5	257		
Kallikrein (pH 8.1)	1.0	33.0	330		
	2.5	38.4	154	43.1	$3.1 \cdot 10^{-5}$
	5.0	40.6	81		
Thrombin (pH 8.1)	2.0	1.87	9.35		
	3.0	2.28	7.60	4.1	$2.4 \cdot 10^{-4}$
	8.0	3.13	3.91		

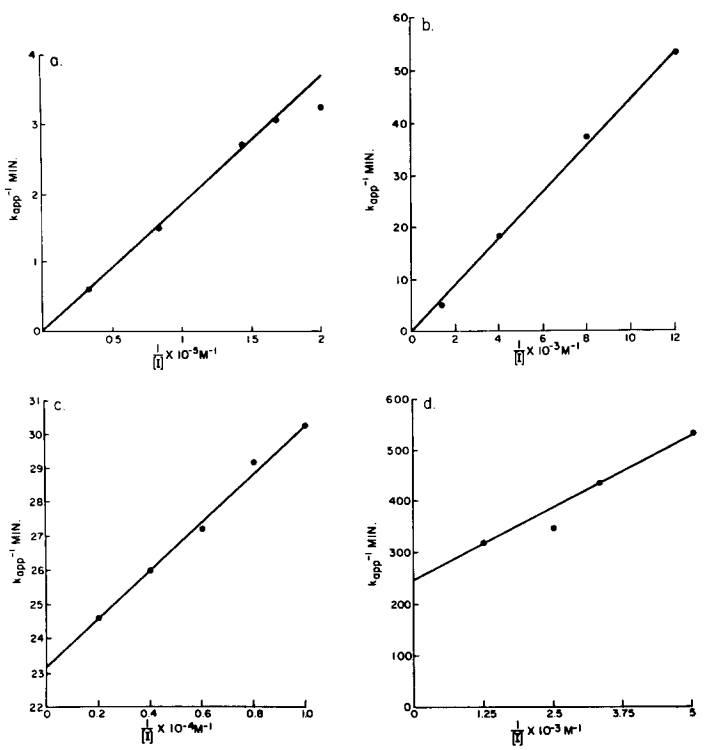

Fig. 1. Plots of $1/k_{app}$ against $1/[I]$ for the irreversible inhibition of α-chymotrypsin (a), trypsin (b), pancreatic kallikrein (c), and thrombin (d) by PDFA. The inhibitor concentrations for chymotrypsin were $5 \cdot 10^{-6}$–$30 \cdot 10^{-6}$ M, for trypsin $0.83 \cdot 10^{-4}$–$7.5 \cdot 10^{-4}$ M, for kallikrein $1 \cdot 10^{-4}$–$5 \cdot 10^{-4}$ M, and for thrombin $2 \cdot 10^{-4}$–$8 \cdot 10^{-4}$ M.

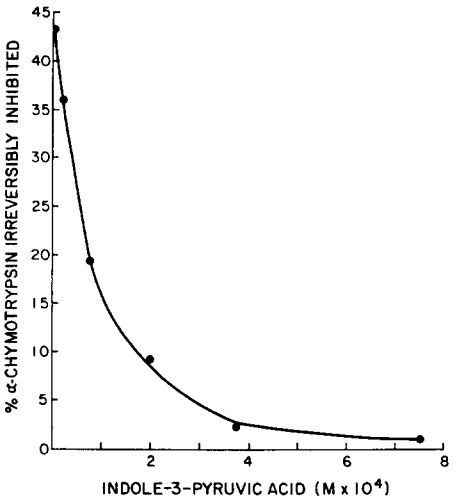

Fig. 2. Effect of indole-3-pyruvic acid (IPyA) on the irreversible inhibition of α-chymotrypsin by PDFA at 37 °C and pH 7.0. The incubation mixtures contained $6.5 \cdot 10^{-6}$ M α-chymotrypsin, $1.2 \cdot 10^{-5}$ M PDFA, 0.05 M $CaCl_2$, 0.025 M imidazole–HCl and various concentrations of IPyA. The reaction was allowed to proceed for 1 min, and then the residual enzyme activity was measured in a rate assay.

acid, a powerful reversible inhibitor of the enzyme[10], could be shown to suppress irreversible inhibition of chymotrypsin by PDFA (Fig. 2).

As mentioned in the Methods section we were unable to obtain kinetic constants for the inhibition of elastase by PDFA. Still we could show that a time-dependent irreversible reaction between elastase and PDFA does indeed occur. For this demonstration, mixtures containing the enzyme and various concentrations of inhibitor were added either immediately to the weighed-out substrate or after a 30-min preincubation period. From Fig. 3 it is evident that preincubation increased considerably the inhibition of the enzyme, a finding which can be explained best by formation of an irreversible enzyme–inhibitor complex.

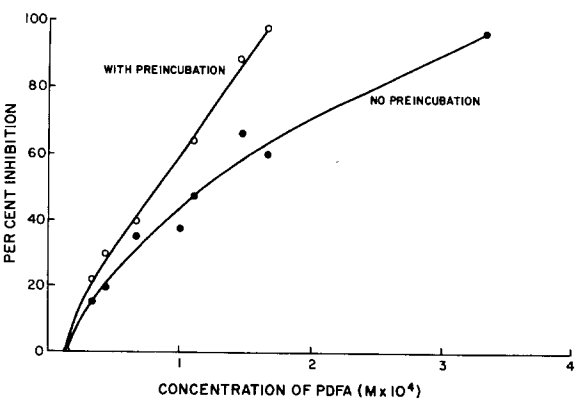

Fig. 3. Inhibition of elastase activity by PDFA with and without 30-min preincubation of enzyme and inhibitor.

DISCUSSION

Our kinetic studies have shown that PDFA is, indeed, a highly effective irreversible inhibitor of α-chymotrypsin, and its potency compares favorably with that of other inhibitory compounds (Table III). From the observations with the competitive inhibitor indole-3-pyruvic acid it appears likely that the active site of α-chymotrypsin is involved in the binding of PDFA, and it is felt that in a first step the dichlorophenoxyacetamide moiety is positioned into the tosyl hole of chymotrypsin and that in a second step the portion of the inhibitor molecule remaining outside the cavity will enter into a covalent bond with a reactive amino acid side chain in the vicinity of the active site. For three inhibitors related to PDFA, Cardinaud and Baker[14] have already demonstrated that sulfonylation of serine is involved in the bonding mechanism, and for one of these compounds they have presented evidence that it is not the active site serine 195 which participates in the reaction, but probably serine 223.

TABLE III

SECOND-ORDER CONSTANTS OF INACTIVATION OF α-CHYMOTRYPSIN FOR VARIOUS IRREVERSIBLE INHIBITORS

Inhibitor	*pH*	*Temperature (°C)*	k^* $(M^{-1} \cdot min^{-1} \times 10^{-2})$	*Reference*
PDFA	7.0	37	541**	—
Diphenylcarbamyl chloride	7.0	25	366	11
Phenylmethanesulfonyl fluoride	7.0	25	149	12
DFP	7.0	25	27	12
L-1-Chloro-4-phenyl-3-tosylamido-2-butanone	6.45	30.5	0.387	13

* k, second-order rate constant of inactivation.
** Value obtained from the curve in Fig. 1a.

Though the inhibitory effect of PDFA is not directed exclusively against α-chymotrypsin, the specificity of the inhibitor remains quite remarkable. The second-order rate constant with α-chymotrypsin (pH 7.0), for example, is more than 200 times greater than with trypsin (pH 8.1), and this can be contrasted with phenylmethanesulfonyl fluoride which is only 55 times more active against chymotrypsin than against trypsin[12]. It should also be noted that thrombin is much more susceptible to inhibition by phenylmethanesulfonylfluoride than by PDFA, the pseudo-first-order rate of inactivation with PDFA at $1 \cdot 10^{-3}$ M (pH 8.1, 37 °C) being only 1/120 of the rate with phenylmethanesulfonylfluoride (pH 8.0, 23 °C)[15].

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