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pH EFFECTS IN PLASMIN-CATALYSED HYDROLYSIS OF α-*N*-BENZOYL-L-ARGININE COMPOUNDS

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Summary

The steady-state kinetics of plasmin (EC 3.4.21.7) catalysed reactions with some α-*N*-benzoyl-L-arginine compounds is investigated in the pH range 5.8—9.0. The results are interpreted in terms of a three-step mechanism, which involves enzyme-substrate complex formation, followed by acylation and deacylation of the enzyme. α-*N*-Benzoyl-L-arginine methyl ester and ethyl ester show the same pH behaviour. The kinetic parameter k_c/K_m is influenced by two groups with pK values of 6.5 and 8.4, respectively. k_c is affected only by the group with pK equal to 6.5 and K_m only by the group with pK equal to 8.4. It is suggested that the group with pK equal to 6.5 is the 1-chloro-3-tosylamido-7-amino-2-heptanone-sensitive histidine residue in the active site and that the group with pK equal to 8.4 is perhaps the α-amino group of the N-terminus in analogy to trypsin and chymotrypsin.

α-*N*-Benzoyl-L-arginine amide is not hydrolysed by plasmin, but proves to be a competitive inhibitor, K_i = 12.8 ± 1.8 mM, pH = 7.8. Also the product α-*N*-benzoyl-L-arginine is a competitive inhibitor, K_i = 26 ± 3.1 mM, pH = 7.8.

Estimates of individual rate constants are compared with similar trypsin data.

Introduction

Plasmin (EC 3.4.21.7) is a proteolytic enzyme of the serine protease class. It is formed from the inactive precursor, plasminogen present in blood plasma. A single diisopropylphosphorofluoridate-sensitive serine [1] and a single Tos-

Abbreviations: Bz-Arg-OEt, α-*N*-benzoyl-L-arginine ethyl ester; Bz-Arg-OMe, α-*N*-benzoyl-L-arginine methyl ester; Bz-Arg-NH_2, α-*N*-benzoyl-L-arginine amide; Bz-Arg-NAn, α-*N*-benzoyl-L-arginine *p*-nitroanilide; Bz-Arg-OH, α-*N*-benzoyl-L-arginine; Tos-Lys-CH_2Cl 1-chloro-3-tosyl-amido-7-amino-2-heptanone.

Lys-CH_2 Cl-sensitive histidine [1] are constituents of the active site. The following three-step mechanism is generally assumed to be a minimal mechanism for serine proteases and has been shown to account also for some plasmin reactions [4—6].

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 + P_1 ; ES_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P_2 \quad (1)$$

This is a Ping Pong BiBi mechanism with water as the second substrate. The kinetic pattern in aqueous solution with the water concentration held constant is however Ordered Uni Bi [2,3]. The substrate, S, which is either an ester or an amide (e.g. a peptide), reacts with the enzyme, E, to form an enzyme-substrate complex, ES_1; the alcohol or amine, P_1, is released and an acyl-enzyme intermediate is formed, ES_2 (acylation), finally this intermediate is decomposed to the acid, P_2, and the free enzyme (deacylation).

In the reaction with no products present the Ordered Uni Bi mechanism leads to the steady-state rate equation

$$v = V/(1 + K_m/[S]) = k_c e/(1 + K_m/[S]) \quad (2a)$$

v = initial velocity; V = maximum velocity; the catalytical constant, $k_c = k_2 k_3/(k_2 + k_3)$; the apparent Michaelis constant, $K_m = K_S k_3/(k_2 + k_3)$, the Michaelis constant $K_S = (k_{-1} + k_2)/k_1$ and thus $k_c/K_m = k_2/K_S$ independent of k_3. The pH dependence of the kinetic parameters can be used to detect ionizing enzyme groups, if they are playing an essential role in the catalytical function of the enzyme [7].

The present work was undertaken to study the pH dependence of plasmin catalysed hydrolysis of three α-*N*-benzoyl-L-arginine compounds, the amide, the methyl ester and the ethyl ester, in order to investigate the catalytically essential ionization in plasmin and to compare the kinetics of amide and ester hydrolysis. It is found that plasmin catalysis is affected by two ionizing groups, one with pK equal to 6.5 and one with pK equal to 8.4. The amide is shown not to be a substrate, but a competitive inhibitor for plasmin.

Materials and Methods

Preparations of human plasminogen were made from 700 to 900 ml of outdated human serum essentially as described by Deutsch and Mertz [8]. The yields were about 4—5 ml plasminogen solution $c \approx 100$ μM (≈ 50 mg) in 0.15 M Tris · HCl buffer, pH 7.8. Human plasmin was prepared by activation of plasminogen on urokinase-substituted Sepharose [9]. The column had a diameter of 15 mm and a length of 28 mm and contained 50.000 P.U. of urokinase. Plasminogen diluted to $c = 10$ μM in 0.15 M Tris · HCl buffer, pH 7.8, was passed through the column at the flow rate 0.1 ml/min at 20° C. 1-ml fractions were collected at 4° C in test tubes each containing 1 ml glycerol. The degree of activation obtained was 95—99%. This plasmin ($c \approx 5$ μM, 50% glycerol/Tris buffer) was stable for several months when kept at −20° C.

Bz-Arg compounds were obtained from Sigma Chemical Co., U.S.A. Urokinase were from Leo A/S, Copenhagen, Denmark. The solvents used were

0.05 M Tris · HCl buffer, 0.1 M NaCl, pH 7.0—9.0 and 0.1 M sodium phosphate buffer, pH 5.8—7.2. A Radiometer pH-meter 4 was used for pH measurements.

The concentrations of plasminogen and plasmin solutions were determined by titration as described earlier [4].

The initial velocities of the plasmin catalyzed hydrolysis of Bz-Arg-OEt, Bz-Arg-OMe and Bz-Arg-NH_2 at different pH values were obtained at substrate concentrations in the range 0.1—3.0 mM. Plasmin concentrations were 0.1 or 8 μM. The reactions were followed by measuring the appearance of Bz-Arg-OH at $\lambda = 253$ nm using a Beckmann DB or Zeiss PM Q II spectrophotometer equipped with a log-converter and a Radiometer REC 61 recorder. The hydrolysis of Bz-Arg-NAn inhibited with Bz-Arg-NH_2 or Bz-Arg-OH at pH 7.8 was measured at $\lambda = 410$ nm. Five substrate concentrations in the range 0.01—0.2 mM and five inhibitor concentrations, 1—30 mM, were used. The plasmin concentration was 1 μM. All experiments were run three times at 25° C.

The initial velocities, v, were taken as the slopes of the tangents to the recorded curves at zero time and the results were fitted to the following equation

$$v/e = k_c/(K_m/[S] + 1) \tag{2b}$$

using a least squares method and assuming equal variance for the velocities, v/e being the absolute velocity independent of the enzyme concentration, e. The calculations were carried out using a Fortran program, which provides values of K_m, k_c, K_m/k_c, k_c^{-1}, the standard error of their estimates, and weighting factors (reciprocal of squares of standard errors) [10]. Inhibition was further analysed by plotting the values of K_m/k_c and k_c against inhibitor concentration to determine the type of inhibition. Values of the inhibition constants and estimates of their accuracy were obtained by making weighted least squares fits to these replots, using the weighting factors supplied by fits to Eqn 2. The pH dependence of a kinetic parameter, P, can be expressed quite generally by Eqn 3 [7],

$$P = P'/[1 + K_A/[H^+] + [H^+]/K_B] \tag{3}$$

where P' is the pH-independent parameter, and K_A and K_B are the acid dissociation constants of two enzymic groups, which are essential for the catalysis ($K_A < K_B$). Provided that K_A is sufficiently smaller than K_B the plot log P versus pH has the slope value +1, when $[H^+] \gg K_B$; 0, when $K_B \ll H^+ \ll K_A$ and −1, when $[H^+] \ll K_A$. K_A and K_B are usually determined from the points of intersection of the tangents with integer slope values, where pH equals either pK_A or pK_B [7]. The number of ionizing groups observable in plasmin and the preliminary values of their ionisation constants were obtained in this way. Final values were obtained by fits to Eqn 4 or Eqn 5. k_c and K_m were each apparently only dependent of one ionizing group, the pK values of which were well separated.

$$P = P'/[1 + K_A/[H^+]]; [H^+] \ll K_B \tag{4}$$

which in fact is the same as Eqn 2.

$$P = P'/[1 + [H^+]/K_B]; [H^+] \gg K_A. \quad (5)$$

Again the weighting factors supplied by the fits to Eqn 2 were used.

Results

The hydrolysis of Bz-Arg-OEt and Bz-Arg-OMe by plasmin was measured in the pH range 5.8—9.0. Values of kinetic parameters and standard errors of their estimates are shown in Tables I and II. At any fixed pH the k_c values are equal, while the K_m value is greater for the methyl ester than for the ethyl ester. Both reactions show the same pH profile. k_c shows dependence on pH only in the lower range and K_m only in the upper range. The values of the pH-independent kinetic parameters and the ionisation constants of catalytically active groups in plasmin, estimated by fits to Eqn 4 or Eqn 5, are shown in Table III. The rates of the catalytic reactions are affected by the ionisation of at least two enzymic groups, one with pK = 6.5 and one with pK = 8.4. Neither the four K_A nor the four K_B values estimated are significantly different. The true K_B value of k'_c and that of k'_c/K'_m may be different, $K_B(k'_c) > K_B(k'_c/K'_m)$, corresponding to a difference in ionisation constant between enzyme-substrate complex and free enzyme, as seen from Table III.

TABLE I

KINETIC PARAMETERS FOR PLASMIN HYDROLYSIS OF Bz-Arg-OEt, 25°C

Buffers: 0.1 M phosphate, pH 5.82—7.20* and 0.05 M Tris · HCl, 0.1 M NaCl, pH 7.01**—8.99.

pH	K_m (mM)	k_c (s^{-1})	k_c/K_m ($mM^{-1} \cdot s^{-1}$)
5.82	0.27 ± 0.13	3.0 ± 0.72	11 ± 0.8
6.03	0.28 ± 0.045	4.1 ± 0.26	15 ± 1.4
6.21	0.27 ± 0.027	4.8 ± 0.18	17 ± 1.0
6.40	0.28 ± 0.019	6.4 ± 0.18	22 ± 0.96
6.46	0.22 ± 0.017	7.2 ± 0.21	33 ± 1.7
6.60	0.20 ± 0.027	7.9 ± 0.37	39 ± 3.5
6.68	0.21 ± 0.012	9.3 ± 0.20	45 ± 1.8
6.77	0.20 ± 0.024	9.6 ± 0.40	47 ± 3.8
6.99	0.19 ± 0.016	11.2 ± 0.32	58 ± 3.3
7.20*	0.19 ± 0.017	12.0 ± 0.37	62 ± 4.3
7.01**	0.20 ± 0.028	11.4 ± 0.61	57 ± 5.6
7.20	0.22 ± 0.030	12.2 ± 0.64	56 ± 5.1
7.40	0.21 ± 0.030	12.1 ± 0.63	58 ± 5.8
7.60	0.23 ± 0.020	12.4 ± 0.40	53 ± 3.1
7.80	0.25 ± 0.033	11.2 ± 0.59	44 ± 3.8
8.00	0.28 ± 0.015	12.1 ± 0.28	44 ± 1.3
8.20	0.32 ± 0.055	12.0 ± 0.91	37 ± 3.5
8.30	0.38 ± 0.039	14.1 ± 0.70	37 ± 2.2
8.40	0.40 ± 0.046	12.8 ± 0.72	32 ± 2.0
8.53	0.51 ± 0.059	13.1 ± 0.81	27 ± 2.1
8.70	0.61 ± 0.18	11.8 ± 1.98	19 ± 2.6
8.80	0.78 ± 0.20	13.8 ± 2.2	17 ± 1.8
8.99	0.98 ± 0.28	13.5 ± 2.5	14 ± 1.3

TABLE II

KINETIC PARAMETERS FOR PLASMIN HYDROLYSIS OF Bz-Arg-OMe, 25°C

Buffers: 0.1 M phosphate, pH 5.8—7.17 and 0.05 M Tris · HCl, 0.1 M NaCl, pH 7.52—9.01.

pH	K_m (mM)	k_c (s^{-1})	k_c/K_m ($mM^{-1} \cdot s^{-1}$)
5.80	0.25 ± 0.056	2.3 ± 0.27	9.1 ± 1.0
6.01	0.37 ± 0.072	4.3 ± 0.39	12 ± 1.1
6.20	0.31 ± 0.038	5.5 ± 0.28	18 ± 1.3
6.60	0.26 ± 0.037	7.6 ± 0.44	29 ± 2.5
6.70	0.28 ± 0.041	8.3 ± 0.47	30 ± 2.6
6.80	0.25 ± 0.043	9.4 ± 0.64	37 ± 4.0
7.00	0.24 ± 0.028	9.7 ± 0.45	40 ± 3.1
7.17	0.30 ± 0.057	10.3 ± 0.86	34 ± 4.1
7.52	0.29 ± 0.046	11.2 ± 0.72	39 ± 3.0
7.83	0.34 ± 0.040	12.0 ± 0.64	35 ± 2.3
8.00	0.36 ± 0.041	12.4 ± 0.65	34 ± 2.2
8.18	0.39 ± 0.055	11.8 ± 0.81	30 ± 2.4
8.40	0.50 ± 0.073	12.1 ± 0.95	24 ± 1.8
8.50	0.57 ± 0.098	13.8 ± 1.8	24 ± 1.9
8.60	0.68 ± 0.16	10.7 ± 1.5	16 ± 1.5
8.65	0.74 ± 0.13	11.6 ± 1.3	16 ± 1.2
8.70	0.77 ± 0.26	12.8 ± 2.8	17 ± 2.3
9.01	4.6 ± 2.5	19.2 ± 9.2	4.2 ± 0.24

Analogous experiments were performed with Bz-Arg-NH_2. Plasmin failed to hydrolyze this compound at a measurable rate. The enzyme concentration was increased to 8 μM, (80 times the one used in ester hydrolysis), and the concentration of Bz-Arg-NH_2 was varied up to 3.0 mM, (15 times the value of K_m for Bz-Arg-OEt at optimal pH). The absorbance of the reaction mixture was measured for more than 1 h, but no reaction was observed at any pH in the range 5.8—9.0. Thus, if Bz-Arg-NH_2 is a plasmin substrate at all, it is a poor substrate. The hydrolysis of Bz-Arg-NAn by plasmin was investigated under

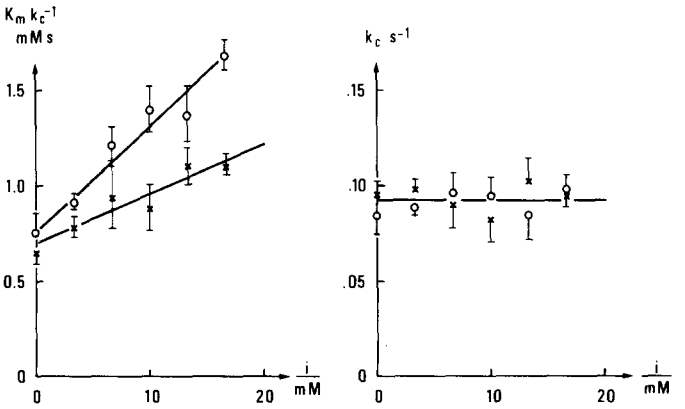

Fig. 1. Inhibitions of plasmin by Bz-Arg-NH_2 and Bz-Arg-OH with Bz-DL-Arg-NAn as substrate, pH 7.8, 25°C. Influence of inhibitor concentration on the kinetic parameters of the plasmin-catalyzed hydrolysis of Bz-Arg-NAn. ○———○, i = Bz-Arg-NH_2 concentration (mM); X———X, i = Bz-Arg-OH concentration (mM). K_m/k_c and k_c and their standard errors were determined at different fixed inhibitor concentrations by fitting to Eqn 2 the results of 15 initial velocity experiments, where the inhibitor concentration was held constant and the substrate concentration was varied in the range 0.01—0.2 mM.

TABLE III

pH-INDEPENDENT VALUES OF KINETIC PARAMETERS, P', AND IONISATION CONSTANTS OF CATALYTICALLY ACTIVE GROUPS IN PLASMIN

Equation fitted to	pH range	Substrate	P'	Ionisation constant of enzymic group	pK
$1/K_m = 1/[K'_m\,(1 + K_A/[H^+])]$	7.2—9.0	Bz-Arg-OEt	$K'_m = 0.195 \pm 0.0022$ mM	$K_A = (4.4 \pm 0.26) \cdot 10^{-9}$	8.36
	7.5—9.0	Bz-Arg-OMe	$K'_m = 0.27 \pm 0.012$ mM	$K_A = (3.5 \pm 0.46) \cdot 10^{-9}$	8.46
$k_c/K_m = k'_c/[K'_m(1 + K_A/[H^+])]$	7.2—9.0	Bz-Arg-OEt	$k'_c/K'_m = 59 \pm 1.4$ mM$^{-1} \cdot$ s^{-1}	$K_A = (3.4 \pm 0.28) \cdot 10^{-9}$	8.47
	7.5—9.0	Bz-Arg-OMe	$k'_c/K'_m = 45 \pm 2.8$ mM$^{-1} \cdot$ s^{-1}	$K_A = (3.5 \pm 0.61) \cdot 10^{-9}$	8.46
$1/k_c = [1 + [H^+]/K_B]/k'_c$	5.8—7.0	Bz-Arg-OEt	$k'_c = 14.5 \pm 19$ s^{-1}	$K_B = (3.4 \pm 0.51) \cdot 10^{-7}$	6.47
	5.8—7.0	Bz-Arg-OMe	$k'_c = 12.5 \pm 1.8$ s^{-1}	$K_B = (4.3 \pm 0.66) \cdot 10^{-7}$	6.37
$K_m/k_c = [1 + [H^+]/K_B]K'_m/k'_c$	5.8—7.0	Bz-Arg-OEt	$k'_c/K'_m = 68 \pm 12$ mM$^{-1} \cdot$ s^{-1}	$KB = (2.6 \pm 0.50) \cdot 10^{-7}$	6.58
	5.8—7.0	Bz-Arg-OMe	$k'_c/K'_m = 50 \pm 5.3$ mM$^{-1} \cdot$ s^{-1}	$K_B = (3.3 \pm 0.43) \cdot 10^{-7}$	6.48

TABLE IV

KINETIC PARAMETERS OF PLASMIN AND TRYPSIN

Enzyme	Bz-Arg- compound	K_m (mM)	k_c (s^{-1})	K_s (K_i) (mM)	k_2 (s^{-1})	k_3 (s^{-1})	pH	°C	Ref.
Plasmin	-OEt	0.25	12	(12)	560	12	7.8	25	
	-OMe	0.34	12	(12)	420	12	7.8	25	
	-NH_2	(12)	≈0	12	≈0	(12)	7.8	25	
	-NAn	0.06	0.09	12	16	0.09	7.8	25	4
	-OH	—	—	26	—	—	7.8	25	
Trypsin	-OEt	0.0043	14.6	(4)	≈13000	15	8.0	25	11
		0.018	19.0		≈4000	19	8.0	30	12
	-OMe		14.4				8.4	25	13
	-NH_2	3.3	0.87	3—5	≈1	≈15	7.9	35	14
		2.5	2.8				8.0	30	15
	-NAn	1.5	0.74	(4)	≈2	≈1.2	8.0	25	12
		0.94	0.61				8.15	15	16
	-OH	—	—	5	—	—	8.0	25	14

influence by Bz-Arg-NH_2. The results are illustrated in Fig. 1. k_c is not affected and K_m increases linearly with increasing Bz-Arg-NH_2 concentration. Bz-Arg-NH_2 thus appears to be a linear competitive inhibitor of plasmin. The value of the inhibition constant estimated was K_i = 12.8 ± 1.8 mM, pH 7.8. Similar experiments where Bz-Arg-OH were used as modifier gave the results also illustrated in Fig. 1. This compound, which is one of the products in the reactions investigated, also inhibits plasmin in the linear competitive way, K_i = 26 ± 3.1 mM, pH 7.8 in consistens with the Ordered Uni Bi mechanism, Eqn 1 [2,3].

Discussion

The pH dependencies found for plasmin suggest the participation of at least two essential groups ionizing in the pH range investigated, one with pK = 6.5 and one with pK = 8.4. The group with pK = 6.5 influences k_c and k_c/K_m. Since k_c/K_m is affected and increases with increasing pH, the group involved is free to ionize in the free enzyme and active in acylation in the unprotonated form [7]. Since k_c is affected and in the same way, the group must ionize in the enzyme-substrate complex, the degradation of which is rate determining. Again the unprotonated form is the active one. It is reasonable to assume that deacylation is rate determining for plasmin-catalysed hydrolysis of Bz-Arg-OEt and Bz-Arg-OMe. The k_c values are equal at the same pH in the entire range and it is very likely that deacylation is the same in the two reactions, while acylation is not.

The group with pK = 6.5 is probably the imidazolyl function of the histidine shown by Groskopf et al. [1] to be inhibited by Tos-Lys-CH_2Cl. Chymotrypsin and trypsin show the same kinetic pattern correlated with a histidine residue [7]. The pK values estimated from the pH dependence of k_c and k_c/K_m are not sufficiently different to determine, whether pK is the same in free plasmin and plasmin-substrate complex or not.

On the alkaline side k_c/K_m and K_m are affected, corresponding to the ionization of a group with pK = 8.4. The group ionize in the free enzyme and is active in acylation in the protonated form. It is not active in deacylation or not free to ionize in the acylated enzyme, since k_c is unaffected. A pK of 8.4 may correspond to ionization of an amino group, a sulfhydryl group or a phenolic hydroxyl group. In chymotrypsin and trypsin there seems to be no doubt that one of the essential residues in catalysis is the -NH_3^+ group of the N-terminal isoleucine [7]. The group with pK = 8.4 in plasmin could, by analogy with chymotrypsin and trypsin, be the free α-amino function of the N-terminal formed in activation. The group is active in enzyme-substrate complex formation and not in deacylation, and an important event in activation could be the formation of an amino group necessary for enzyme-substrate complex formation.

Bz-Arg-NH_2 proved to be a competitive inhibitor of plasmin (Fig. 1). The dissociation constant of the enzyme-inhibitor complex was estimated to $K_i \approx$ 12 mM, pH 7.8. The same value was found for the Michaelis constant, K_s, in plasmin hydrolysis of Bz-Arg-NAn, where $K_s = k_{-1}/k_1$, as $k_2 \ll k_{-1}$ [4]. Since plasmin shows the same affinity for two Bz-Arg compounds with such different substituents, substituent effects are probably unimportant in the formation of

the first complex in the reaction. The value $k_{-1}/k_1 = 12$ mM may therefore be valid also for the ester substrates. Assuming $k_{-1} \gg k_2$ and $k_{-1}/k_1 = 12$ mM makes it possible to estimate k_2 and k_3. For hydrolysis of Bz-Arg-OEt at pH 7.8:

$$K_m = (k_{-1} + k_2)k_3/[(k_2 + k_3)k_1] = 0.25 \text{ mM}$$

or

$$k_2 = 47\, k_3$$

since

$$k_c = k_2k_3/(k_2 + k_3) = 12 \text{ s}^{-1}$$

the final result is

$$k_3 = 12.3 \text{ s}^{-1} \text{ and } k_2 = 564 \text{ s}^{-1}$$

this confirmes, that deacylation is the rate-determining step.

If the relation $k_{-1} \gg k_2$ does not hold, we would get $k_2 > 564$ s^{-1} and $k_3 = k_c = 12$ s^{-1}.

Table IV shows estimated values of the kinetic constants of the reactions of plasmin and trypsin with Bz-Arg compounds. The similarity of plasmin to trypsin is evident, but the data also point out some differences. Towards the esters trypsin shows 3—4 times greater binding affinity $\approx K_s^{-1}$, and approx. 10 times faster acylation rate, $\approx k_2$, than does plasmin; the same is shown with the amide, which plasmin failed to hydrolyse at an observable rate, very likely because of an extremely slow acylation rate. Many inhibitors are bound more tightly to trypsin than to plasmin [17,18]. This is also true of the ethyl and *p*-nitrophenyl esters of *p*-guanidinobenzoate for which at the same time the acylation rates are faster in trypsin than in plasmin catalysis [5]. Thus plasmin appears as a generally less efficient catalyst, compared with trypsin and it is to be expected that the specificity of plasmin is more narrow than that of trypsin as seen, e.g. with fibrinogen as the substrate [19].

In the reaction of plasmin with Bz-Arg-NAn the liberation of *p*-nitroaniline has been observed to occur before the rate-determining step [4]. The same k_3 value would therefore be expected for the anilide and the ester reaction. But k_3 in the ester reaction is found to be approx. 100 times as big as that of the anilide reaction. If both reactions proceed via the mechanism of Eqn 1, an explanation could be that Bz-Arg-NAn strains plasmin into an unfavourable conformation, the deacylation of which is slow, compared to the one attained with the esters. The data indicates that this may also apply to trypsin.

The difference between K_i values of Bz-Arg-OH and the K_s values of the other compounds both in plasmin and trypsin may be accounted for by assuming here $K_i = k_3/k_{-3}$, rather than $K_i = k_{-1}/k_1$ (Eqn 1).

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References

1 Groskorf, W.R., Hsieh, B., Summaria, L. and Robbins, K.C. (1969) J. Biol. Chem. 244, 359—365
2 Plowman, K.M. (1972) Enzyme Kinetics, p. 44, McGraw-Hill, New York
3 Plowman, K.M. (1972) Enzyme Kinetics, p. 138, McGraw-Hill, New York
4 Christensen, U. and Müllertz, S. (1974) Biochim. Biophys. Acta 334, 187—198
5 Chase, J. and Shaw, E. (1969) Biochemistry 8, 2212—2224
6 Sodetz, J.M. and Castellino, F.J. (1972) Biochemistry 11, 3167—3171
7 Laidler, K.J. and Bunting, P.S. (1973) The Chemical Kinetics of Enzyme Action, pp. 142—162, Clarendon Press, Oxford
8 Deutsch, D.G. and Mertz, E.T. (1970) Science 170, 1095—1096
9 Wiman, B. and Wallén, P. (1973) Eur. J. Biochem. 36, 25—38
10 Cleland, W.W. (1967) Adv. Enzymol. 29, 1—32
11 Inagami, T. and Sturtevant, J.M. (1960) J. Biol. Chem. 235, 1019—1023
12 Yoshida, N., Sasaki, A. and Inoue, H. (1971) FEBS Lett. 15, 129—132
13 Baines, N.J., Baird, J.B. and Elmore, D.T. (1964) Biochem. J. 90, 470—476
14 Chevallier, J. and Yon, J. (1966) Biochim. Biophys. Acta 122, 116—126
15 Wang, S. and Carpenter, F.H. (1968) J. Biol. Chem. 243, 3702—3710
16 Erlanger, B.F., Kokowsky, N. and Cohen, W. (1961) Arch. Biochem. Biophys. 95, 271—278
17 Markwardt, F., Walsmann, P., Stürzebecher, J., Landmann, H. and Wagner, G. (1973) Pharmazie 28, 326—330
18 Markwardt, F., Richter, P., Stürzebecher, J., Wagner, G. and Walsmann, P. (1974) Acta. Biol. Med. Germ. 33, K1—K7
19 Robbins, K.C. and Summaria, L. (1970) Methods in Enzymology (Perlmann, G.E. and Lorand, L., eds), Vol. 19, p. 198, Academic Press, New York