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STEADY-STATE KINETICS OF PLASMIN- AND TRYPSIN-CATALYSED HYDROLYSIS OF A NUMBER OF TRIPEPTIDE-*p*-NITROANILIDES

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Summary

The steady-state kinetics of plasmin- (EC 3.4.21.7) and trypsin-catalysed (EC 3.4.21.4) hydrolysis of Bz-L-Phe-Val-Arg-pNA, Bz-D-Phe-Val-Arg-pNA, L-Phe-Val-Arg-pNA, D-Phe-Val-Arg-pNA and D-Val-Leu-Lys-pNA were investigated in the pH range 6—9. The pH dependences of the kinetic parameters correspond with the effects of catalytically essential ionizations in the enzymes, except for reactions with L- and D-Phe-Val-Arg-pNA, in which protonation of the NH_2-terminal α-amino groups ($pK = 7.0$) shows some inhibitory effect.

The reactions of plasmin and trypsin with the *p*-nitroanilides show k_c values similar to those normally found with specific ester substrates, indicating that the deacylation steps of the reactions are rate determining.

Introduction

A large number of tripeptide-*p*-nitroanilide compounds that are substrates for serine proteinases have been synthesized in recent years (e.g. Refs. 1 and 2). Knowledge of the specificity of various enzymes towards the substrates is essential. It is nevertheless common to use as a measure of specificity the (so-called) relative reaction rate (the ratio of the rates of hydrolysis of two different substrates measured at the same arbitrarily chosen concentration). Since the reaction rate of an enzyme-catalysed process is not a linear function

Abbreviations: Bz-L-Phe-Val-Arg-pNA, α-*N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine-4-nitroanilide; L-Phe-Val-Arg-pNA, L-phenylalanyl-L-valyl-L-arginine-4-nitroanilide; Bz-D-Phe-Val-Arg-pNA, α-*N*-benzoyl-D-phenylalanyl-L-valyl-L-arginine-4-nitroanilide; D-Phe-Val-Arg-pNA, D-phenylalanyl-L-valyl-L-arginine-4-nitroanilide; D-Val-Leu-Lys-pNA, D-valyl-L-leucyl-L-lysine-4-nitroanilide; Bz-Arg-OEt, α-*N*-benzoyl-L-arginine ethyl ester; Bz-Arg-OMe, α-*N*-benzoyl-L-arginine methyl ester.

of the substrate concentration, relative reaction rates are dependent on the substrate concentration and, therefore, poor measures of the specificity of the enzyme. The kinetic parameters of the reactions are the main source of such information. This paper presents the kinetic parameters for plasmin- (EC 3.4.21.7) and trypsin-catalysed (EC 3.4.21.4) hydrolysis of a number of tripeptide-*p*-nitroanilides obtained at various pH values.

Materials and Methods

Preparations of human plasminogen were made from 1—1.2 l outdated human plasma, essentially as described by Deutsch and Mertz [3]. Human plasmin was prepared by conversion of NH_2-terminal glutamic acid plasminogen on a column of urokinase-substituted Sepharose 4B [4,5] the eluate was concentrated by ultrafiltration to approx. 2.5 μM (25% glycerol, 0.05 M Tris-HCl, pH 7.8).

Bovine trypsin 'Crystalline Trypsin Novo' was provided as a gift from Novo (Copenhagen, Denmark). Stock solutions (approx. 2.5 μM) were prepared in 1 mM HCl.

The compounds Bz-L-Phe-Val-Arg-pNA (S-2160), L-Phe-Val-Arg-pNA (S-2170), Bz-D-Phe-Val-Arg-pNA (S-2174), D-Phe-Val-Arg-pNA (S-2178) and D-Val-Leu-Lys-pNA (S-2251) were kindly provided as gifts from Dr. G. Claeson (Kabi Peptide Research, Göteborg, Sweden) or from Kabi Vitrum (Copenhagen, Denmark).

The buffers used were 0.025 M Tris-HCl (pH 7.0—8.5), 0.025 M Tris-HCl, 0.01 M $CaCl_2$ (pH 7.0—8.5), 0.05 M Tris/maleate, 0.1 M NaCl (pH 5.53—9.00) and 0.05 M Tris/maleate, 0.05 M NaCl, 0.02 M $CaCl_2$ (pH 6.05—8.99).

Methods. The enzyme concentrations of plasmin and trypsin stock solutions were each determined by titration with 4′-nitrophenyl-4-guanidinobenzoate [6], and with polyvalent proteinase inhibitor [7].

Stock solutions of D-Val-Leu-Lys-pNA were made in water. Stock solutions of the other substrates were made in water-containing dimethylformamide ($\leqslant$2%, v/v), normally approx. 25 μmol substrate was dissolved in 200 μl dimethylformamide and then 10 ml water was added.

The kinetic parameters of plasmin- and trypsin-catalysed hydrolysis of each of the substrates at various pH values were determined. The measurements and calculations were made in an analogous manner to some described earlier [5]. The reactions were followed by measuring the appearance of 4-nitroaniline at $\lambda = 410$ nm using a Beckman 35 or Zeiss PM QII spectrophotometer. The enzyme concentrations in the reaction mixtures were 50 nM plasmin or 25 nM trypsin.

Each pair of kinetic parameters was determined from a fit to Eqn. 1 [5,8]:

$$v/e_0 = k_c/(1 + K_m/s) \quad (1)$$

of at least 24 initial velocities, v, measured at eight substrate concentrations, s, in the range 0.05—2.5 mM. All experiments were run at least three times at 25°C.

The pH dependence of the kinetic parameters may be used to detect ionizing enzymic groups playing an essential role in the catalytic function of the

enzyme [9]. The acid dissociation constants of such enzymic groups were determined as previously described [5].

Titrations of the substrates were performed using a Radiometer ABU 13 Autoburette, a PHM 62 Standard pH meter and TTT 60 Titrator.

Results and Discussion

The kinetic parameters of plasmin- and trypsin-catalysed hydrolysis of D-Val-Leu-Lys-pNA, Bz-L-Phe-Val-Arg-pNA, Bz-D-Phe-Val-Arg-pNA, L-Phe-Val-Arg-pNA and D-Phe-Val-Arg-pNA were determined at various pH values, 25° C. The results are shown in Table I—VI. In some of the series of experiments the use of concentrations of the substrate high enough to determine values of K_m (and k_c) was prohibited by the solubility of the substrate. In such cases k_c/K_m values were determined only.

The pH dependencies found previously for plasmin-catalysed hydrolysis of Bz-Arg-OEt and Bz-Arg-OMe suggested the participation of two essential groups of plasmin ionizing in the pH range 6—9, one with pK = 6.5 and one with pK = 8.4. The present results confirm this. The pH dependencies of the kinetic parameter k_c/K_m of plasmin-catalysed hydrolysis of D-Val-Leu-Lys-pNA and Bz-L-Phe-Val-Arg-pNA, respectively, show the same pH profile and the pK values determined are equal to those previously obtained for plasmin (Table VII) [5]. In the pH range investigated the catalytic constant, k_c, of D-Val-Leu-Lys-pNA is constant, whereas that of Bz-L-Phe-Val-Arg-pNA is decreasing at high pH corresponding to the effect of an ionizing enzymic group with pK = 8.4. The ionizations of the two enzymic groups account satisfactorily for the pH variations of the kinetic parameters of Bz-D-Phe-Val-Arg-pNA, but not for those of D- and L-Phe-Val-Arg-pNA. In both of these reactions protonation of the α-amino group of the phenylalanyl residue of the substrate seems to inhibit the reaction. The pK values of the α-amino groups of D- and L-Phe-Val-Arg-

TABLE I

KINETIC PARAMETERS FOR PLASMIN HYDROLYSIS OF D-Val-Leu-Lys-pNA

Temperature, 25°C. Buffers: 0.05 M Tris/maleate, 0.1 M NaCl (pH 5.53—7.00), and 0.05 M Tris-HCl, 0.1 M NaCl (pH 7.25—9.00). The values are given ± S.E. of their estimates.

pH	K_m (mM)	k_c (s^{-1})	k_c/K_m ($s^{-1} \cdot mM^{-1}$)
5.53	—	—	4.3 ± 0.1
6.05	0.53 ± 0.03	13.1 ± 0.3	25 ± 0.9
6.30	0.32 ± 0.02	13.6 ± 0.3	43 ± 1.5
6.50	0.21 ± 0.01	14.2 ± 0.2	68 ± 1.8
6.80	0.19 ± 0.01	14.4 ± 0.2	77 ± 2.0
7.00	0.14 ± 0.01	13.8 ± 0.2	97 ± 3.8
7.25	0.14 ± 0.01	12.2 ± 0.3	87 ± 4.9
7.60	0.15 ± 0.01	15.2 ± 0.2	102 ± 3.5
8.00	0.22 ± 0.02	13.5 ± 0.5	60 ± 4.1
8.25	0.25 ± 0.02	15.1 ± 0.6	61 ± 3.8
8.50	0.34 ± 0.03	16.3 ± 0.7	48 ± 3.0
8.73	0.34 ± 0.08	13.0 ± 1.2	37 ± 5.3
9.00	0.45 ± 0.06	13.8 ± 0.8	31 ± 2.2

TABLE II

KINETIC PARAMETERS FOR PLASMIN HYDROLYSIS OF Bz-L-Phe-Val-Arg-pNA

Temperature, 25°C. Buffers: 0.01 M phosphate, pH 6.0—6.6, and 0.015 M Tris/HCl, pH 7.0—8.8.

pH	K_m (mM)	k_c (s^{-1})	k_c/K_m ($s^{-1} \cdot mM^{-1}$)
6.00	—	—	2.6 ± 0.2
6.10	—	—	3.7 ± 0.4
6.60	—	—	10.0 ± 0.2
7.00	1.20 ± 0.20	14.0 ± 1.7	12.0 ± 0.5
7.20	1.10 ± 0.11	12.2 ± 0.9	11.5 ± 0.4
7.60	0.90 ± 0.11	11.8 ± 1.0	13.5 ± 0.5
8.00	0.69 ± 0.04	8.5 ± 0.3	12.4 ± 0.3
8.25	0.80 ± 0.08	8.7 ± 0.5	10.3 ± 0.3
8.50	0.90 ± 0.09	8.0 ± 0.6	9.2 ± 0.3
8.65	0.90 ± 0.25	6.7 ± 1.5	7.7 ± 0.5
8.80	1.00 ± 0.32	5.6 ± 1.3	5.2 ± 0.4

TABLE III

KINETIC PARAMETERS FOR PLASMIN HYDROLYSIS OF L-Phe-Val-Arg-pNA, Bz-D-Phe-Val-Arg pNA AND D-Phe-Val-Arg-pNA

Temperature, 25°C. Buffer: 25 mM Tris/HCl, pH 7.0—8.5.

Substrate	pH	K_m (mM)	k_c (s^{-1})	k_c/K_m ($s^{-1} \cdot mM^{-1}$)
L-Phe-Val-Arg-pNA	7.0	0.66 ± 0.09	1.4 ± 0.1	2.2 ± 0.2
	7.6	1.90 ± 0.09	5.2 ± 0.2	2.7 ± 0.1
	8.5	—	—	1.4 ± 0.1
Bz-D-Phe-Val-Arg-pNA	7.2	—	—	4.6 ± 0.3
	7.6	1.98 ± 0.08	13.2 ± 0.4	6.7 ± 0.1
	8.5	—	—	2.2 ± 0.1
D-Phe-Val-Arg-pNA	7.2	0.48 ± 0.06	4.6 ± 0.3	9.6 ± 0.7
	7.6	0.62 ± 0.06	10.5 ± 0.6	17.0 ± 0.7
	8.5	1.0 ± 0.2	11.0 ± 1.1	11.0 ± 1.0

TABLE IV

KINETIC PARAMETERS FOR TRYPSIN HYDROLYSIS OF D-Val-Leu-Lys-pNA

Temperature, 25°C. Buffer: 0.05 M Tris/maleate, 0.05 M NaCl, 0.02 M $CaCl_2$, pH 6.05—8.99.

pH	K_m (mM)	k_c (s^{-1})	k_c/K_m ($s^{-1} \cdot mM^{-1}$)
6.05	2.10 ± 0.20	16.3 ± 0.8	8 ± 0.2
6.54	0.91 ± 0.06	14.0 ± 0.5	14 ± 0.5
6.83	0.70 ± 0.12	11.4 ± 1.1	17 ± 1.3
7.03	0.78 ± 0.04	21.9 ± 0.6	28 ± 0.7
7.23	0.67 ± 0.04	21.6 ± 0.6	32 ± 0.9
7.59	0.56 ± 0.04	22.1 ± 0.7	40 ± 1.4
7.80	0.49 ± 0.03	22.1 ± 0.7	45 ± 1.5
7.96	0.60 ± 0.12	23.7 ± 2.4	41 ± 4.5
8.01	0.47 ± 0.04	21.5 ± 0.9	46 ± 2.7
8.25	0.61 ± 0.06	25.7 ± 1.2	42 ± 2.4
8.99	0.50 ± 0.08	22.1 ± 1.6	45 ± 3.8

TABLE V

KINETIC PARAMETERS FOR TRYPSIN HYDROLYSIS OF Bz-L-Phe-Val-Arg-pNA

Temperature, 25°C. Buffers: 0.01 M phosphate, pH 6.0—6.5 and 0.025 M Tris-HCl, 0.02 M $CaCl_2$, pH 7.0—9.0.

pH	K_m (mM)	k_c (s^{-1})	k_c/K_m ($s^{-1} \cdot mM^{-1}$)
6.00	—	—	55 ± 5
6.50	0.110 ± 0.020	15.8 ± 1.3	138 ± 12
7.00	0.080 ± 0.011	23.7 ± 1.0	300 ± 30
7.20	0.066 ± 0.006	28.2 ± 0.8	430 ± 28
7.60	0.046 ± 0.003	29.5 ± 1.3	644 ± 25
8.00	0.052 ± 0.004	33.3 ± 0.8	644 ± 30
8.25	0.055 ± 0.003	33.0 ± 0.7	600 ± 27
8.50	0.043 ± 0.003	31.2 ± 0.7	716 ± 42
9.00	0.038 ± 0.002	24.9 ± 0.4	650 ± 29

TABLE VI

KINETIC PARAMETERS FOR TRYPSIN HYDROLYSIS OF L-Phe-Val-Arg-pNA, Bz-D-Phe-Val-Arg-pNA AND D-Phe-Val-Arg-pNA

Temperature, 25°C. Buffer: 25 mM Tris/HCl, pH 7.0—8.5.

Substrate	pH	K_m (mM)	k_c (s^{-1})	k_c/K_m ($s^{-1} \cdot mM^{-1}$)
L-Phe-Val-Arg-pNA	7.0	0.30 ± 0.05	11.0 ± 1.1	37 ± 2.3
	7.6	0.28 ± 0.02	34.1 ± 0.9	123 ± 5.0
	8.5	0.25 ± 0.01	30.9 ± 0.4	122 ± 2.3
Bz-D-Phe-Val-Arg-pNA	7.0	0.19 ± 0.01	17.9 ± 0.3	101 ± 3.6
	7.6	0.20 ± 0.05	33.4 ± 1.6	167 ± 14.9
	8.5	0.14 ± 0.01	29.6 ± 0.6	214 ± 10.5
D-Phe-Val-Arg-pNA	7.0	0.09 ± 0.01	15.8 ± 0.3	184 ± 11.6
	7.6	0.08 ± 0.01	19.2 ± 0.5	257 ± 19.1
	8.5	0.06 ± 0.01	18.8 ± 0.3	315 ± 26.5

TABLE VII

pH INDEPENDENT VALUES OF THE KINETIC PARAMETER, k_c'/K_m', AND pK VALUES OF CATALYTICALLY ACTIVE GROUPS IN PLASMIN AND TRYPSIN IN THE pH RANGE 6.0—9.0

The values were determined from fits to $[k_c/K_m = k_c'/K_m'/(1 + [H^+]/K_a + K_b/[H^+])]$ of the plasmin k_c/K_m values of Tables I and II and from fits to $[k_c/K_m = k_c'/K_m'/(1 + [H^+]/K_a)]$ of the trypsin k_c/K_m values of Tables IV and V.

Enzyme	Substrate	k_c'/K_m' ($s^{-1} \cdot mM^{-1}$)	pK_a	pK_b
Plasmin	D-Val-Leu-Lys-pNA	110 ± 14	6.50	8.43
	Bz-L-Phe-Val-Arg-pNA	14 ± 1.3	6.22	8.66
Trypsin	D-Val-Leu-Lys-pNA	48 ± 1.4	6.88	—
	Bz-L-Phe-Val-Arg-pNA	686 ± 29	7.09	—

pNA were each determined by titration. Both are 7.0. The results (Table III) indicate that the protonated form of L-Phe-Val-Arg-pNA is deacylated only slowly, if at all, from plasmin since the catalytic constant and the apparent Michaelis constant which are both dependent on k_3, the deacylation rate constant, ($k_c = k_2k_3/(k_2 + k_3)$ and $K_m = K_sk_3/(k_2 + k_3)$ [5]) both decrease, whereas the parameter k_c/K_m which is independent of k_3 ($k_c/K_m = k_2/K_s$) does not change from pH 7.6 to pH 7.0. In contrast to this protonation of D-Phe-Val-Arg-pNA presumably decreases both the acylation and deacylation rates of that reaction. Protonation of the α-amino group of D-Val-Leu-Lys-pNA (pK = 7.2) shows no influence on the reaction with plasmin.

The pH profiles of trypsin-catalysed hydrolysis of D-Val-Leu-Lys-pNA and of Bz-L-Phe-Val-Arg-pNA (Table VII) correlate well with those of other trypsin-catalysed reactions reported in the literature (See for examples [10]). Protonation of the α-amino group of D-Val-Leu-Lys-pNA (Table IV) and of the D-Phe-Val-Arg-pNA (Table VI) shows no influence on their respective reactions with trypsin, but protonation of L-Phe-Val-Arg-pNA seems to decrease both the acylation and the deacylation rates of that reaction. The results obtained on the four Phe-Val-Arg-pNA substrates (Tables V and VI) seem to indicate that their reactions with trypsin each involves the same specific orientation of the phenylalanyl residue, so that after complex formation the α-substituent of an L-epimer is in contact with non-polar areas of the trypsin active site, whereas that of an D-epimer is in contact with the solvent. If so, then complex formation with Bz-L-Phe-Val-Arg-pNA is thus accompanied by a favourable transfer of the non-polar benzoyl group from the polar solvent to a non-polar environment with correspondent high value of k_c/K_m; that group is unfavourably kept in contact with the solvent when a Bz-D-Phe-Val-Arg-pNA-trypsin complex is formed with correspondent low value of k_c/K_m. Further if the α-substituent of the substrate is an amino group then binding of the D-epimer is favoured (high value of k_c/K_m) and protonation of the α-amino group predominantly effects binding of the L-epimer, which involves the transfer of the polar group from the solvent to a non-polar area of the enzyme active site. Protonation of the amino group of the L-epimer leads to a decrease of the value of k_c/K_m. Some differences presumably exist between plasmin and trypsin in the S_4—S_3 area of the active sites since for example the parameter k_c/K_m of the L-Phe-Val-Arg-pNA-plasmin reaction is not affected when the α-amino group of the substrate is protonated.

All of the Phe-Val-Arg-pNA compounds are much better substrates of trypsin than of plasmin. The reverse is true of D-Val-Leu-Lys-pNA. For plasmin the values of the kinetic parameters of D-Val-Leu-Lys-pNA and Bz-Arg-OEt are alike and the Phe-Val-Arg-pNA compounds show values of the apparent Michaelis constants greater than, but values of the catalytic constants similar to those of D-Val-Leu-Lys-pNA and Bz-Arg-OEt [5]. Also trypsin shows values of the catalytic constants of all of the *p*-nitroanilide substrates of the same order of magnitude as those normally found for specific ester substrates (15—50 s^{-1}) [10]. The results presented here thus indicate that the deacylation step is rate determining in trypsin- and plasmin-catalysed hydrolysis of a number of *p*-nitroanilide substrates and therefore that a rate-determining breakdown of a tetrahedral intermediate in such reactions as suggested by Petkov [11] is not a general phenomenon.

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