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KINETIC STUDIES OF UROKINASE-CATALYSED HYDROLYSIS OF 5-OXO-L-PROLYLGLYCYL-L-ARGININE 4-NITROANILIDE

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Summary

The enzymic properties of urokinase (EC 3.4.21.31) were studied. The kinetic parameters of hydrolysis of 5-oxo-Pro-Gly-Arg-NA were determined in the pH range 5—9, at 25°C and 37°C. The reaction is affected by only one ionizing group of urokinase with pK 7.15 (25°C) and pK 6.82 (37°C). The results indicate that 5-oxo-Pro-Gly-Arg-NA is a good model substrate for studies of the conversion of plasminogen to plasmin. The K_m values of the urokinase-catalysed hydrolysis of plasminogen and 5-oxo-Pro-Gly-Arg-NA are of the same order of magnitude. Plasmin catalyses the hydrolysis of 5-oxo-Pro-Gly-Arg-NA, but the K_m value is several hundred times that of urokinase. Urokinase is shown not to react with good plasmin substrates, such as Bz-Arg-OEt and D-Val-Leu-Lys-NA, but is linearly competitively inhibited by 6-aminohexanoic acid and *trans*-4-aminomethylcyclohexane-1-carboxylic acid.

Introduction

Urokinase (EC 3.4.21.31) is a serine proteinase which catalyses the conversion of plasminogen to plasmin. The bond hydrolysed is the Arg-560-Val-561 peptide bond of plasminogen. The sequence of amino acid residues prior to the scissile bond is -S-S-Cys-557-Pro-558-Gly-559-Arg-560- [1,2]. The compound 5-oxo-Pro-Gly-Arg-NA is a urokinase substrate with amino acid residues P_3-P_2-P_1 analogous to those of plasminogen.

Abbreviations: 5-oxo-Pro-Gly-Arg-NA, 5-oxo-L-prolylglycyl-L-arginine 4-nitroanilide; D-Val-Leu-Arg-NA, D-valyl-L-leucyl-L-arginine 4-nitroanilide; D-Val-Leu-Lys-NA, D-valyl-L-leucyl-L-lysine 4-nitroanilide; Bz-Ile-Glu-Gly-Arg-NA, α-*N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine 4-nitroanilide; Bz-Arg-OEt, α-*N*-benzoylarginine ethyl ester; Ac-Lys-NA, α-*N*-acetyllysine 4-nitroanilide; CBZ-Gly-Gly-Arg-4-methoxy-2-naphthylamide, α-*N*-carbobenzoxyglycyl-glycyl-L-arginine 4-methoxy-2-naphthylamide.

The present paper describes the results of a number of kinetic experiments designed to investigate the enzymic properties of urokinase and to compare them with those of plasmin. Particular attention was paid to the reaction of urokinase with 5-oxo-Pro-Gly-Arg-NA.

Materials and Methods

Human plasmin was prepared as previously described [3].

Urokinase was the 'Reagent' preparation from LEO (Copenhagen, Denmark). A concentration of urokinase 1.81 ± 0.05 μM was the mean value resulting from 18 experiment using 4'-nitrophenyl-4-guanidinobenzoate for titration of solutions containing 10 000 Ploug U/ml urokinase (i.e. 1 mol = $5.5 \cdot 10^{12}$ Ploug U).

The compunds 5-oxo-Pro-Gly-Arg-NA (S-2444), D-Val-Leu-Lys-NA (S-2251), Bz-Ile-Glu-Gly-Arg-NA (S-2222) and *trans*-4-aminomethylcyclohexane-1-carboxylic acid were kindly provided as gifts from Kabi Vitrum A/S (Copenhagen, Denmark). L-Lysine-HCl was obtained from Merck (Darmstadt, F.R.G.), 6-aminohexanoic acid from Fluka (Buchs, Switzerland) and Bz-Arg-OEt from Calbiochem (La Jolla, CA, U.S.A.).

The solvents used were 0.05 M Tris/maleate buffers, 0.1 M NaCl, pH 5.—9. A Radiometer pH meter 62 was used for pH measurements.

The initial velocities of the urokinase-catalysed reactions were obtained at (at least) eight substrate concentrations in the range 0.01—0.5 mM of 5-oxo-Pro-Gly-Arg-NA at different pH values, 25°C and 37°C; 0.1—1.0 mM of Bz-Ile-Glu-Gly-Arg-NA at pH 7.6, 25°C and 37°C; 0.1—3 mM of D-Val-Leu-Lys-NA, pH 7.6, 25°C and 0.1—3 mM of Bz-Arg-OEt, pH 7.6, 37°C. The reaction of urokinase with 5-oxo-Pro-Gly-Arg-NA was further studied in the presence of 6-aminohexanoic acid (10, 25, 50, 100 and 200 mM), *trans*-4-aminomethylcyclohexane-1-carboxylic acid (1.67, 3.33, 6.67, 13.3 and 25 mM), L-lysine (50, 100 and 250 mM) all at pH 8.0, 37°C and 1.2 mM D-Val-Leu-Lys-NA and 3 mM Bz-Arg-OEt both at pH 7.6, 25°C. Stock solutions of urokinase were 181 nM and the concentration of urokinase in the reaction mixtures was 9 nM. The reaction of plasmin and 5-oxo-Pro-Gly-Arg-NA was studied at eight substrate concentrations in the range 0.05—1.5 mM at a plasmin concentration of 66 nM, pH 7.8, 25°C. The reactions were followed by measuring the appearance of 4-nitroanilide at λ = 410 nm (or Bz-Arg at λ = 253 nm) using a Beckman Model 35 spectrophotometer. All experiments were run three times.

The initial velocities, v ($\mu M \cdot s^{-1}$), were calculated from the slopes of the tangents to the recorded curves at zero time and the results (at least 24 pairs of ([s], v)-values) were fitted to the Michaelis equation (Eqn. 1),

$$v/e = k_c/(1 + K_m/[s]) \quad (1)$$

using a least-squares method and assuming equal variance for the velocities, v/e, being the absolute velocity independent of the enzyme concentration, e. [s] is the substrate concentration. The calculations were carried out using a Fortran program, which provides values of the kinetic parameters, K_m, k_c, k_c/K_m, k_c^{-1}, K_m/k_c, the standard error of their estimates, and weighting factors [4]. Inhibition was further analysed by plotting the values of K_m (i) and k_c (i) (K_m and k_c

obtained in the presence of an inhibitor) against inhibitor concentrations to determine type of inhibition. Values of the inhibition constants and estimates of their accuracy were obtained by making weighted least-squares fits to the equations corresponding to the observed type of inhibition, i.e. for linear competitive inhibition, K_m (i) = $K_m(1 + i/K_i)$ and k_c (i) = k_c. The values of the kinetic parameters and their weighting factors supplied by fits to Eqn. 1 were used.

The theory of the pH dependence of a serine proteinase predicts [5], that if K_{es} is the acid dissociation constant of an enzymic group of a serine proteinase in that of the two enzyme-substrate complexes, the degradation of which is rate determining, and that group is essential for catalysis in the unprotonated form, then the pH dependence of the kinetic parameter, k_c, is

$$k_c = k'_c/(1 + [H^+]/K_{es}) \quad (2)$$

k'_c is the pK_{es} independent parameter. The corresponding pH dependence of the kinetic parameter, k_c/K_m, is

$$k_c/K_m = (k'_c/K'_m)/(1 + [H^+]/K_e) \quad (3)$$

where K_e is the acid dissociation constant of the enzymic group in the free enzyme [5]. In the pH range 5—9 only one ionizing group of urokinase with a pH dependence equal to that described above was observed. Values of the ionization constants, K_e and K_{es}, were obtained from fits to Eqn. 2 and Eqn. 3. Again the weighting factors supplied by the fits to Eqn. 1 were used.

Results and Discussion

The hydrolysis of 5-oxo-Pro-Gly-Arg-NA catalysed by urokinase was studied in the pH range 5—9 at 25° C and 37° C. Values of the kinetics parameters, k_c and k_c/K_m, are plotted against pH in Figs. 1 and 2. In the pH range 5—8.5 the reaction is affected by one ionizing group of urokinase. As seen from Table I there is a difference between the K_{es} value of k'_c and the K_e value of k'_c/K'_m, corresponding to a difference in acid dissociation constant between enzyme-

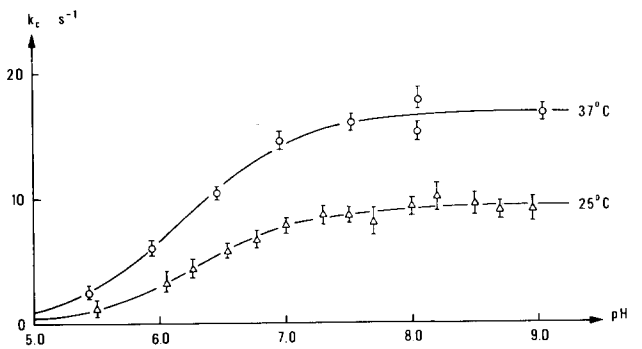

Fig. 1. The pH dependence of the kinetic parameter k_c for urokinase-catalysed hydrolysis of 5-oxo-Pro-Gly-Arg-NA at 25° C (△) and 37° C (○). The curves shown are those given by Eqn. 2 using the values of k'_c and K_{es} of Table I.

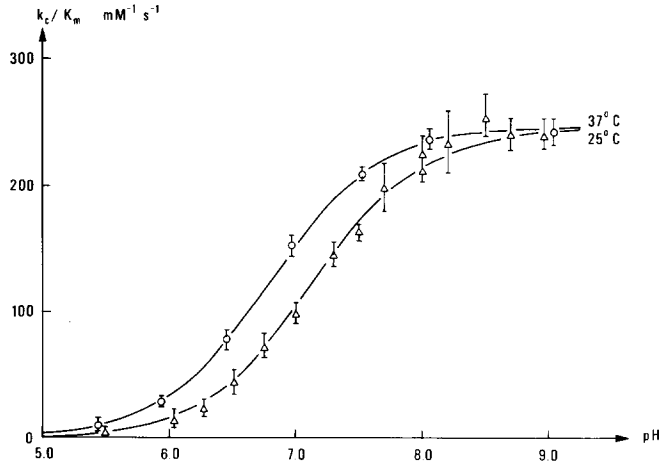

Fig. 2. The pH dependence of the kinetic parameter k_c/K_m for urokinase-catalysed hydrolysis of 5-oxo-Pro-Gly-Arg-NA at 25°C (△) and 37°C (○). The curves shown are those given by Eqn. 3 using the values of k'_c/K'_m and K_e of Table I.

substrate complex and free enzyme. Since the pK values are in the range 6—7 and there is good agreement between the change of pK observed when the temperature is raised from 25°C to 37°C, and that calculated from the heat of ionization of an imidazolyl group ($\Delta pK = -0.2$ pH unit) [6], the ionizing group of urokinase is probably the imidazolyl function of the catalytic site histidine characteristic for serine proteinases.

It is seen (Figs. 1 and 2) that a possible high pH effect on urokinase requires that the corresponding pK value be greater than 9.5. The high pK for plasmin is 8.4 [3,7].

The kinetic parameters for urokinase-catalysed hydrolysis of a number of peptide substrates were determined. The results are presented in Table II. It is seen that urokinase hydrolyses a number of substrates, but only 5-oxo-Pro-Gly-Arg-NA is a real good substrate. Urokinase is one of the serine proteinases which requires P_1-Arg or P_1-Lys substrates. There are many indications that the serine proteinases each possesses a catalytic site similar to that of the other

TABLE I

pH-INDEPENDENT VALUES OF THE KINETIC PARAMETERS FOR UROKINASE-CATALYSED HYDROLYSIS OF 5-OXO-PRO-GLY-ARG-NA, AND pK VALUES OF THE UROKINASE GROUP ESSENTIAL FOR CATALYSIS, WHICH IONIZES IN THE pH RANGE 5.0—9.0

The kinetic parameters, P (= k_c/K_m, k_c), were determined at a number of pH values. The acid dissociation constants of the urokinase group, K (= K_e, K_{es}), and the pH-independent kinetic parameters, P', were determined from fits to $P = P'/(1 + H^+/K)$. Free urokinase: K_e; urokinase-substrate complex: K_{es}.

Temperature (°C)	k'_c (s^{-1})	K'_m (μM)	k'_c/K'_m ($s^{-1} \cdot mM^{-1}$)	pK_e	pK_{es}
25	9.3 ± 0.3	37 ± 1	255 ± 9	7.15 ± 0.06	6.30 ± 0.07
37	16.7 ± 0.8	67 ± 3	248 ± 12	6.82 ± 0.12	6.17 ± 0.14

TABLE II

KINETIC PARAMETERS OF UROKINASE-CATALYSED HYDROLYSIS OF A NUMBER OF PEPTIDE SUBSTRATES

Substrate	k_c/K_m ($s^{-1} \cdot mM^{-1}$)	k_c (s^{-1})	K_m (mM)	pH	Temperature (°C)
5-Oxo-Pro-Gly-Arg-NA	255 ± 9	9.3 ± 0.5	0.04 ± 0.005	8—9	25
5-Oxo-Pro-Gly-Arg-NA	248 ± 10	17 ± 0.8	0.07 ± 0.005	8—9	37
Bz-Ile-Glu-Gly-Arg-NA *	3.6 ± 0.2	3.6 ± 0.3	1.0 ± 0.3	7.5	25
Bz-Ile-Glu-Gly-Arg-NA *	4.4 ± 0.2	5.8 ± 0.6	1.3 ± 0.2	7.5	37
D-Val-Leu-Arg-NA	0.65 ± 0.08	—	>5	8.0	25
CBZ-Gly-Gly-Arg-4-methoxy-2-naphthyl-amide [11]	—	—	0.49	8.75	22
Ac-Lys-NA [12]	0.143	0.8 ± 0.05	5.8 ± 0.4	8.5	37

* This substrate is a mixture (1:1) of Bz-Ile-Gly-Gly-Arg-NA and Bz-Ile-Glu-(4-methyl ester)-Gly-Arg-NA.

serine proteinases and that they all probably employ a common mechanism to catalyse the hydrolysis of their substrates. Therefore sites other than the catalytic site may be responsible for individual enzyme specificities. Plasmin possesses such second sites [8,9], presumably urokinase does not. The kinetic parameters for urokinase-catalysed hydrolysis of 5-oxo-Pro-Gly-Arg-NA (Table II) are of the order of magnitude as those for urokinase-catalysed conversion of plasminogen [10]. This tripeptide substrate is thus as good a substrate of urokinase as is plasminogen. Indeed urokinase shows a narrow specificity even towards tripeptide substrates (Table II), and therefore the catalytic site probably solely determines its specificity. The P_3-Pro residue of the substrate seems to be very important and derivatives of Pro-Gly-Arg should be valuable models in studies of urokinase reaction.

One of the objections of this work was to compare the enzymic properties of urokinase and plasmin. Table III summarizes the results. Urokinase failed to

TABLE III

KINETIC PARAMETERS FOR UROKINASE AND PLASMIN-CATALYSED HYDROLYSIS OF A NUMBER OF PEPTIDE SUBSTRATES AND DISSOCIATION CONSTANTS (K_i FOR SOME REVERSIBLE COMPETITIVE INHIBITORS OF UROKINASE AND PLASMIN

Buffer: 0.05 M Tris-HCl, 0.1 M NaCl, pH 8.0, 25°C.

Compound	Enzyme	k_c (s^{-1})	k_c/K_m ($s^{-1} \cdot mM^{-1}$)	K_i (mM)
5-Oxo-Pro-Gly-Arg-NA	urokinase	9.4 ± 0.5	255 ± 9	—
	plasmin	8.8 ± 0.6	8.6 ± 0.9	—
D-Val-Leu-Lys-NA	urokinase	0	0	*
	plasmin	13.5 ± 0.5	60 ± 4	— [7]
Bz-Arg-OEt	urokinase	0	0	*
	plasmin	12.1 ± 0.3	44 ± 1.3	— [3]
6-Aminohexanoic acid	urokinase	—	—	32 ± 3 (37°C)
	plasmin	—	—	58 ± 4 [8]
trans-4-Aminomethylcyclo-hexane-1-carboxylic acid	urokinase	—	—	5 ± 0.5 (37°C)
	plasmin	—	—	16 ± 2 [8]

* These substrates did not inhibit urokinase.

hydrolyse Bz-Arg-OEt and D-Val-Leu-Lys-NA at a measurably rate. Further when the hydrolysis of 5-oxo-Pro-Gly-Arg-NA by urokinase was investigated in the presence of each of these plasmin substrates the kinetic parameters of that reaction was not affected. The catalytic site of urokinase thus does not bind Bz-Arg-OEt or D-Val-Leu-Lys-NA. D-Val-Leu-Arg-NA is a poor urokinase substrate. 5-Oxo-Pro-Gly-Arg-NA is a poor plasmin substrate.

The effects of 6-aminohexanoic acid, L-lysine and *trans*-4-aminomethylcyclohexane-1-carboxylic acid on urokinase were studied using as substrate 5-oxo-Pro-Gly-Arg-NA. 6-Aminohexanoic acid and *trans*-4-aminomethylcyclohexane-1-carboxylic acid were found to be competitive inhibitors of urokinase, binding more efficiently to the catalytic site of urokinase than to that of plasmin. L-Lysine was found not to inhibit urokinase. In contrast to plasmin, urokinase was otherwise not affected by these amino acids [8].

The substrate specificity of urokinase is narrow and much different from that of plasmin. That allows for the assessment of plasmin in the presence of urokinase. If, however, urokinase is to be assessed in the presence of plasmin, hydrolysis of 5-oxo-Pro-Gly-Arg-NA by plasmin occurs and should be minimized, e.g. by performing the experiment at pH 9.0.

References

1 Wiman, B. and Wallén, P. (1975) Eur. J. Biochem. 58, 539—547
2 Sottrup-Jensen, L., Zajdel, M., Claeys, H., Petersen, T.E. and Magnusson, S. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2577—2581
3 Christensen, U. (1975) Biochim. Biophys. Acta 397, 459—467
4 Cleland, W.W. (1967) Adv. Enzymol. 29, 1—32
5 Laidler, K.J. and Bunting, P.S. (1973) The Chemical Kinetics of Enzyme Action, pp. 142—162, Claredon Press, Oxford
6 Dixon, M. and Webb, E.C. (1964) Enzymes, pp. 143—144, Longmans, London
7 Christensen, U. and Ipsen, H.-H. (1979) Biochim. Biophys. Acta 569, 177—183
8 Christensen, U. (1978) Biochim. Biophys. Acta 526, 194—201
9 Christensen, U. (1979) in The Physiological Inhibitors of Coagulation and Fibrinolysis (Collen, D., Wiman, B. and Vestraete, M., eds.), pp. 137—144, Elsevier/North-Holland Biochemical Press
10 Christensen, U. (1977) Biochim. Biophys. Acta 481, 638—647
11 Bigbee, W.L., Weintraub, H.B. and Jensen, R.H. (1978) Anal. Biochem. 88, 114—122
12 Petkov, D., Christova, E., Pojarlieff, I. and Stambolieva, N. (1975) Eur. J. Biochem. 51, 33—42