THE SUBSTRATE SPECIFICITY OF GUINEA-PIG PLASMA KALLIKREIN

G. Holman, J.S. Lowe, J.S. Morley and M.J. Smithers

Imperial Chemical Industries Limited, Pharmaceuticals Division,
Alderley Park, Macclesfield, Cheshire, England.

Received August 23, 1968

The isolation by Habermann(1966a) of two kinin-containing fragments, PKFL and PKFS, from a peptic digest of highly purified bovine kininogen, apparently ended speculation (Rocha e Silva, 1960; Elliott, 1963) that an ester linkage is involved in the attachment of kinin to the rest of the kininogen molecule; PKFL was identified as the linear tetradecapeptide, H-Met-Lys-BK-Ser-Val-Gln-OH, where BK represents the bradykinin sequence, and PKFS as the hexadecapeptide, H-Met-Lys-BK-Ser-Val-Gln-Val-Met-OH (Habermann, 1966b). There are however discrepancies between natural PKFL and the synthetic tetradecapeptide. Pierce (1968) has suggested that these may be accounted for by the presence in natural PKFL of an ester bond between the C-terminal arginine residue of bradykinin and the adjacent serine residue; such a formulation requires the presence of an additional amino-terminal serine residue in bovine kininogen, which is in fact more consistent with the determinations of Suzuki, Iwanaga, Nagasawa and Sato (1966). In this communication we report studies on the kinetics of the reaction between guinea-pig kallikrein and a series of synthetic substrates. The results support the concepts of Pierce in indicating specificity of kallikrein towards an ester linkage of the type envisaged in the kininogens.

Previous work from these laboratories (Davies and Lowe, 1963) has shown that guinea-pig plasma kallikrein, which occurs in the 7S γ-globulins,

behaves like other kallikreins in splitting off kinin from kininogen and having no effect on other protein substrates (casein, haemoglobin, fibrin). The kallikreins possess esterolytic activity towards esters of N^{α}-benzoyl- or -p-tosyl-L-arginine, but do not hydrolyse lysine methyl ester or N^{α}-benzoyl-L-argininamide (Contzen, Holtz, and Raudonat, 1959; Habermann, 1959; Werle and Kaufmann-Boetsch, 1959; Webster and Pierce, 1961; Prado, Prado, and Brandi, 1962). The only evidence of proteolytic activity is the claim that horse urinary kallikrein hydrolyses protamine sulphate (Brandi, Mendes, Paiva, and Prado, 1965).

Materials and Method. The kinetic studies were carried out at 37° using a Radiometer pH Stat. A solution of the substrate under test (various concentrations) in 2 ml. of 0.005M phosphate buffer (prepared by adding 50 ml. of 0.15M saline and 28 ml. of water to 2 ml. of 0.2M phosphate buffer) was adjusted, if necessary, to the required pH, and equilibrated. Guinea-pig plasma kallikrein (Davies and Lowe, 1963) (same preparation in all experiments) in 0.005M phosphate buffer (1 ml.) of the required pH was then added, and the titration with standard sodium hydroxide (at appropriate concentration) was allowed to proceed for a maximum of 15 min. The initial velocity of the reactions at different substrate concentrations was determined by the mathematical correction procedure of Allgranati (1963), or from the tangent of the curve at time zero. Michaelis Constants were determined graphically from a plot of $^{s}/_{v}$ against s.

Seryl, threonyl, and tyrosyl esters of lysine (cmpds 21-26) were prepared as follows: (a) N^{α}-t-butoxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysine was condensed with N-acetyl-L-serine, -threonine, or -tyrosine amides by the use of N N'-dicyclohexylcarbodi-imide (1 mol.) in pyridine -acetone (1:1) (Morley, 1965); (b) the t-butoxycarbonyl protecting group was cleaved with trifluoroacetic acid at 15°; (c) the resulting trifluoroacetates were coupled with p-nitrophenyl acetate or benzoate (2 mol.) in dimethylformamide and triethylamine (1 mol.) at

4°; (d) the N^{ϵ}-protecting group was removed by hydrogenolysis in methanol containing N-hydrochloric acid (2 mol.). The N^{α}-tosyl or -benzoyl esters of arginine or lysine (cmpds 3-8) were prepared by esterification of the appropriate N^{α}-tosyl or -benzoyl arginine or lysine with thionyl chloride in methanol or ethanol. The remaining N^{α}-acylated methyl esters of lysine were prepared by hydrogenolysis of the appropriate N^{α}-acylated-N^{ϵ}-benzyloxy-carbonyl-L-lysine methyl ester, in turn prepared by acylation of N^{ϵ}-benzyloxy-carbonyl-L-lysine methyl ester. With the exception of compounds 7, 8, 10, 13 and 14, all compounds used were crystalline; their constitution and purity were established by i.r., p.m.r., and elementary analysis, by t.l.c. in four solvent systems, and by paper or thin-layer electrophoresis.

Results (see Table). Like other plasma kallikreins, our guinea-pig preparation did not hydrolyse lysine methyl or ethyl esters (cmpds. 1 & 2); in contrast, the N^{α}-acylated lysine esters (cmpds. 4, 6 & 8) were very good substrates for the enzyme. The optimum pH for the reaction between the enzyme and N^{α}-tosyl-L-arginine or -lysine methyl ester was determined by the colorimetric method of Roberts (1958), and found to be pH 8.6. However, all the substrates reported in this study were hydrolysed spontaneously at this pH, so Michaelis Constants were determined at lower pH's where the effect was of no significance. Comparison of the results with cpds. 3-8 shows that the Km values for lysine esters are close to those of analogous arginine esters. Since lysine is more convenient to handle synthetically, the remainder of our studies were carried out with lysine esters only. The results with cpds. 9-20 show that the lowest Km values (and hence highest affinity for the enzyme) are obtained when the N^{α}-substituent is naphthoyl. The results with cmpds. 21-26 show that the enzyme can hydrolyse lysine esters of serine and tyrosine, but not esters of threonine.

Table. The hydrolysis of lysine and arginine esters by guinea-pig plasma kallikrein

No.	Compound	Km(M)	pH
1.	Lys-OMe, 2HCl	Not substrates	
2.	Lys-OEt, 2HCl		
3.	N^{α}-tosyl-Arg-OMe, HCl	2.0×10^{-3}	7.6
4.	N^{α}-tosyl-Lys-OMe, HCl	4.7×10^{-3}	7.6
5.	N^{α}-tosyl-Arg-OEt, HCl	2.0×10^{-3}	7.6
6.	N^{α}-tosyl-Lys-OEt, HCl	7.3×10^{-3}	7.6
7.	N^{α}-benzoyl-Arg-OMe, HCl	1.8×10^{-4}	7.6
8.	N^{α}-benzoyl-Lys-OMe, HCl	8.4×10^{-4}	7.6
9.	N^{α}-p-methylbenzoyl-Lys-OMe, HCl	6.8×10^{-4}	7.6
10.	N^{α}-p-chlorobenzoyl-Lys-OMe, HCl	7.0×10^{-4}	7.6
11.	N^{α}-p-methoxybenzoyl-Lys-OMe, HCl	3.6×10^{-4}	7.6
12.	N^{α}-benzenesulphonyl-Lys-OMe, HCl	5.1×10^{-3}	7.6
13.	N^{α}-phenylacetyl-Lys-OMe, HCl	1.6×10^{-3}	7.6
14.	N^{α}-phenoxyacetyl-Lys-OMe, HCl	7.0×10^{-4}	7.6
15.	N^{α}-cyclohexylcarbonyl-Lys-OMe, HCl	1.3×10^{-3}	7.6
16.	N^{α}-1-naphthoyl-Lys-OMe, HCl	1.6×10^{-4}	7.6
17	N^{α}-2-naphthoyl-Lys-OMe, HCl	1.1×10^{-4}	7 6
18.	N^{α}-1-naphthylacetyl-Lys-OMe, HCl	6.8×10^{-4}	7.6
19.	N^{α}-acetyl-Lys-OMe, HCl	3.1×10^{-3}	7.6
20.	N^{α}-n-hexoyl-Lys-OMe, HCl	1.8×10^{-4}	7.6
21.	N^{α}-acetyl-O-(N^{α}-benzoyl-lysyl)-serine amide, HCl	5.6×10^{-4}	7.6
22.	N^{α}-acetyl-O-(N^{α}-acetyl-lysyl)-serine amide, HCl	4.2×10^{-4}	7.6
23.	N^{α}-acetyl-O-(N^{α}-benzoyl-lysyl)-tyrosine amide, HCl	1.4×10^{-3}	7.0
24.	N^{α}-acetyl-O-(N^{α}-acetyl-lysyl)-tyrosine amide, HCl	8.4×10^{-4}	7.0
25.	N^{α}-acetyl-O-(N^{α}-acetyl-lysyl)-threonine amide, HCl	Not substrates	
26	N^{α}-acetyl-O-(N^{α}-benzoyl-lysyl)-threonine amide, HCl		

Discussion. In designing model compounds to see whether an enzyme will hydrolyse the ester bond between the carboxyl group of lysine or arginine and the hydroxyl group of serine, threonine, or tyrosine, it is necessary to take steps to ensure that the lysine or arginine residue remains attached to the oxygen of the hydroxyl group and does not undergo the well known O to N shift. In the compounds used in this study (Nos. 21-26) this shift has been prevented by acetylation of the serine, threonine or tyrosine α-amino group. Probably the most significant result is our finding that guinea-pig kallikrein is able to hydrolyse lysine esters of serine (compounds 21 & 22) but not analogous threonine esters (compounds 25-26). Our results with compounds 3-8 indicate that a similar conclusion will apply to arginine esters of serine, and thus support Pierce's view that the kallikreins liberate kinins from kininogen by acting on an ester bond formed between the C-terminal arginine residue of the kinin and an adjacent serine residue.

The lysine esters of tyrosine (compounds 23 & 24) were also substrates for the enzyme, but pH 7.0 had to be used for the kinetic studies with these compounds because of extensive auto-hydrolysis at higher pH's. Change of acetyl for benzoyl (as the N^{α}-lysine substituent) in the serine compounds (nos. 21 & 22) has apparently little effect on the Km; in the tyrosine compounds (nos. 23 & 24), the effect is opposite to that seen in the methyl esters (nos. 8 & 19).

References

Allgranati, I.D., Biochem. Biophys. Acta. 73, 152 (1963).
Brandi, C.M.W., Mendes, J., Paiva, A.C.M., and Prado, E.S , Biochem. Pharmac. 14, 1665 (1965).
Contzen, C., Holtz, P., and Raudonat, H.W., Naturwissen, 46, 402 (1959).
Davies, G.E., and Lowe, J.S., Br. J. Pharmac. Chemother. 21, 491 (1963).
Elliott, D.F., Ann. N.Y. Acad. Sci. 104, 35 (1963).
Habermann, E., Arch. exp. Path Pharmak. 236, 492, (1959).
Habermann, E. In: "Hypotensive Peptides", edited by E.G. Erdös, N. Back, F. Sicuteri, and A.F. Wilde. New York: Springer-Verlag, p. 116 (1966a).

Habermann, E., Arch. exp. Path. Pharmak. 253, 474 (1966b).
Morley, J.S. In: "Peptides. Proc. 6th European Symp., Athens, September 1963", edited by L. Zervas. Oxford: Pergamon, p. 351 (1965).
Pierce, J.V., Fed. Proc. 27, 52 (1968).
Prado, E.S., Prado, J.L., and Brandi, C.M.W., Arch. int. Pharmacodyn. 137, 358 (1962).
Roberts, P.S., J. biol. Chem. 232, 285 (1958).
Rocha e Silva, M. In: "Polypeptides Which Affect Smooth Muscle and Blood Vessels", edited by M. Schachter. Oxford: Pergamon, p. 210 (1960).
Suzuki, T., Iwanaga, S., Nagasawa, S., and Sato, T. In: "Hypotensive Peptides", edited by E.G. Erdös, N. Back, F. Sicuteri, and A.F. Wilde. New York: Springer-Verlag, p. 149 (1966).
Webster, M.E. and Pierce, J.V., Proc. Soc exptl. Biol. N.Y., 107, 186 (1961).
Werle, E. and Kaufmann-Boetsch, B., Naturwiss. 46, 559 (1959).