ACTIVE SITE TITRATION OF PIG PANCREATIC KALLIKREIN WITH *p*-NITROPHENYL *p'*-GUANIDINOBENZOATE

F. FIEDLER, B. MÜLLER and E. WERLE

Institut für Klinische Chemie und Klinische Biochemie der Universität, 8 München 2, Nussbaumstr. 20, Germany

Revised version received 17 May 1972

1. Introduction

Kallikrein (EC 3.4.4.21; the enzyme from porcine pancreas is understood throughout this paper) forms acyl-enzymes in the reactions with diisopropyl fluorophosphate [1] or cinnamoyl and indoleacryloyl imidazoles [2]. Concerning the hydrolytic mechanism of ester cleavage by kallikrein, studies on the hydrolysis of a number of arginine esters with different alcohol moieties failed to reveal the operation of an acyl-enzyme mechanism [3]. In the present report, properties of *p*-nitrophenyl *p'*-guanidinobenzoate (*p*NPGB) as a kallikrein substrate are described. This compound allows the demonstration of acyl-enzyme formation by monitoring the liberation of *p*-nitrophenol. Similar to other enzyme systems [4–6], *p*NPGB proved a useful reagent for the active site titration of kallikrein. The present work also leads to a revised value for the molecular weight of the enzyme.

2. Materials and methods

*p*NPGB-HCl was obtained from Cyclo Chemical Corp. *p*-Nitrophenol, a Merck p.a. product, was twice recrystallized from toluene. A sample of this material was further recrystallized from water. Kallikrein was purified from prepurified preparations from pig pancreastic autolysates generously supplied by Farbenfabriken Bayer AG by the procedure of Fritz et al. [7] modified by application of a buffer gradient. The material obtained was treated with sialidase [7] and separated on DEAE-cellulose into the kallikrein A and B [8] which were further purified by rechromatography and gel filtration on Sephadex G-50. The specific activity of the preparations was determined with α-*N*-benzoyl-L-arginine ethyl ester as substrate as described in [9].

Active site titrations of kallikrein were conducted on a Cary 15 spectrophotometer equipped with a 0–0.1 slide wire and cuvette holders thermostated at 25.0°. The sample cuvette of 1 cm light path contained 1.93 ml 0.1 M veronal buffer, pH 8.3, 1 mM in EDTA [10], which had been filtered through a Sartorius membrane filter SM 11309 of 0.1 μm pore diameter. 20 μl of a 4–10 mM solution of *p*NPGB in acetonitrile/water (4:1 v/v) were added, and the absorbance at 402 nm was recorded for several minutes. Then 50 μl of an aqueous solution of kallikrein (about 4 nmoles) were added. In general, every third experiment was conducted in an inverse manner, starting the reaction by the addition of substrate. Experiments aiming at the determination of the acylation constant were run on a Gibson–Durrum stopped flow spectrophotometer equipped with a cuvette of 2 cm light path, thermostated at 25°. One syringe contained a solution of kallikrein B (about 2 μM) in the above veronal buffer and the other an aqueous solution of *p*NPGB.

3. Results and discussion

On addition of kallikrein to a solution of *p*NPGB, an initial rapid liberation ("burst") of nitrophenol followed by a steady-state reaction occurs (fig. 1). Such a biphasic nitrophenol production is accounted

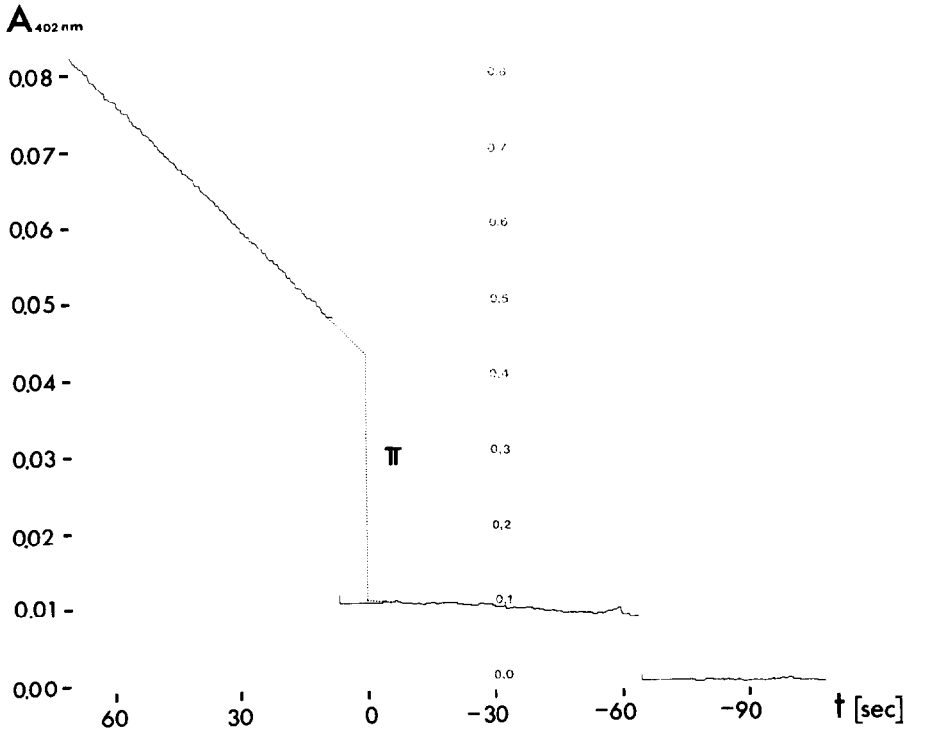

Fig. 1. Reaction of kallikrein A (1.87 μM) with *p*NPGB (100 μM) at pH 8.3. At −65 sec the substrate solution was added and at 0 sec the reaction was started by the addition of enzyme. The dotted line shows the extrapolation back to zero time to obtain the burst π.

for by the reaction scheme

$$E + S \underset{}{\overset{K_s}{\rightleftarrows}} ES \xrightarrow{k_2} P_1 + ES^* \xrightarrow{k_3} P_2 + E,$$

the "burst" reflecting the initial formation of acyl-enzyme ES* (see [11]). This experimental observation thus establishes the operation of an acyl-enzyme mechanism in the kallikrein catalyzed hydrolysis of an ester substrate.

The quantitative treatment of the reaction for the case $[S] \gg [E]_0$ and $k_2 \gg k_3$ applying here has been given by Bender et al. [12,13]. From the first order acylation phase followed on the Gibson–Durrum instrument with [S] ranging from 40 to 100 μM, values of $k_2 = 18 \pm 7$ sec^{-1} and $K_s = 81 \pm 56$ μM ($n = 4$) were obtained for kallikrein B. There is no dependence of the postburst nitrophenol production rate on [S] in the concentration range examined in the experiments carried out with the Cary instrument, indicating $[S] \gg \gg K_m$. "Nonspecific nitrophenol production" [4,5] is excluded, since a preparation of kallikrein B inhibited by 48% (substrate: α-*N*-benzoyl-L-arginine ethyl ester) by controlled treatment with diisopropyl fluorophosphate exhibited a steady-state hydrolysis rate of *p*NPGB 47% slower than native enzyme. Thus the observed rate of *p*-nitrophenol production (after a nearly negligible correction for spontaneous hydrolysis of *p*NPGB) equals k_3, it being $(1.76 \pm 0.06) \cdot 10^{-2}$ sec^{-1} for kallikreins A and B. k_3 is much higher than the values reported for the hydrolysis of *p*NPGB by trypsin, thrombin, or plasmin [5], and is about as large as k_3 with chymotrypsin [4]. $K_m = K_s \cdot k_3/(k_2 + k_3)$ is calculated as 79 ± 63 nM for kallikrein B.

In spite of the rather high value of k_3, back extrapolation to zero time to obtain the burst π is easily possible (fig. 1). As $k_2 \gg k_3$ and $[S] \gg K_m$, the concentration of kallikrein active sites may be taken directly from π after applying a small correction for changes in absorbance caused by the addition of absorbing reagent solutions or their dilution. The results of a number of titrations of different batches of

Table 1

Titration of different batches of kallikreins A and B with *p*NPGB. $[E]_0$, concentration of enzymatically active kallikrein in the cuvette; n, number of titrations conducted; $[E]_T$, concentration of active sites obtained by titration.

	Specific activity (U/mg protein)	$[E]_0$ (U/ml)	n	$\pi \pm \sigma$ (10^{-3} absorbance units)	$[E]_T$ (μM)	Catalytic center activity (min^{-1})
Kallikrein A	240	13.4	7	32.2 ± 0.46	1.87	7 150
Kallikrein B	234	11.3	9	26.6 ± 0.21	1.54	7 370
Kallikrein B	262	13.7	6	32.2 ± 0.14	1.87	7 330
Kallikrein B	300	15.3	5	37.1 ± 0.26	2.15	7 120
Kallikrein B	308	11.6	6	28.7 ± 0.46	1.66	6 980

kallikrein of different specific activities are compiled in table 1. The enzyme solutions used in the first three experiments had been filtered through membrane filters. This treatment decreased the initial specific activities by about 10%. Therefore, filtration was omitted in the last two experiments. Titration results obtained with *p*NPGB concentrations between 40 and 100 μM were identical, as is to be expected because of the very low K_m. Identical results were also obtained in inverse titrations. The small standard error of π demonstrates the very satisfactory reproducibility of the measurements.

Because of the importance of the molar absorbance of *p*-nitrophenol for the evaluation of the titration results, it has been redetermined under the conditions of the titration experiments. At the absorbance maximum at 402 nm a value of 17,230 ± 110 $M^{-1} cm^{-1}$ (n = 8) at pH 8.3 was obtained, in excellent agreement with data calculated from the literature [4,14]. Both recrystallized *p*-nitrophenol sample had identical absorbance. The presence of 0.8% (v/v) acetonitrile or of kallikrein in a concentration equimolar to that of *p*-nitrophenol did not influence absorbance at 402 nm.

The mean value of the catalytic center activity of kallikrein B in α-*N*-benzoyl-L-arginine ethyl ester hydrolysis, 7200 ± 92 min^{-1}, is identical to that of kallikrein A. It was not possible to increase the specific activity of kallikreins A or B beyond a limit of 300 ± 10 U/mg protein by further purification operations. This value, in combination with the titration results and the evidence for a single active site in kallikrein [1], allows a calculation of the molecular weight of the protein moiety of the kallikrein molecule which results as 24,000 ± 860 for both forms of the enzyme. With an average value of 7% for the carbohydrate content of kallikrein [7], the molecular weight emerges as 25,700 ± 920.

Based on a value of 35,000 obtained from gel filtration experiments [15], which is confirmed by Takami [16] who found 34,000, the molecular weight of sialic acid free kallikrein has been calculated from the amino acid and carbohydrate composition as 33,400 to 33,800 [7]. It is well known, however, that during gel filtration glyceroproteins tend to behave as proteins with a higher than the true molecular weight [17].

Several ultracentrifugation studies on the molecular weight of kallikrein are reported in the literature. Habermann [18] obtained a value of 23,000 to 24,000. Moriya revised his earlier result of 33,000 [19] in a recent paper to 25,200 [20]. According to the method of Cohn and Edsall [21], a value of 0.721 ml/g can be calculated for the partial specific volume $\bar{\nu}$ of kallikrein from its amino acid and carbohydrate composition [7] and the specific volumes of the residues compiled in [21–23]. If one recalculates the molecular weight determined by Scholtan [7] using $\bar{\nu} = 0.721$ in place of 0.749, a value of 26,100 (instead of 29,000) results. Thus, the divergent ultracentrifugation results of the different authors appear to converge now at a value around 25,000. This is in good agreement with the molecular weight of kallikrein derived from the present active site titration data.

The molecular weight of the protein moiety of kallikrein determined here is close to that of other pancreatic serine proteinases [24] to which kallikreins A and B evidently bear a close resemblance also in this respect.

Acknowledgements

Our thanks are due to the Institut für Physiologische Chemie und Physikalische Biochemie and to the Pharmakologisches Institut der Universität München for permission to use their Cary 15 and Gibson–Durrum instruments, respectively. This work was sponsored by Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 51).

References

[1] F. Fiedler, B. Müller and E. Werle, European J. Biochem. 10 (1969) 419.
[2] F. Fiedler, B. Müller and E. Werle, FEBS Letters 22 (1972) 1.
[3] E. Werle and F. Fiedler, Biochem. J. 115 (1969) 4 P.
[4] T. Chase, Jr. and E. Shaw, Biochem. Biophys. Res. Commun. 29 (1967) 508.
[5] T. Chase, Jr. and E. Shaw, Biochemistry 8 (1969) 2212.
[6] J.B. Baird and D.T. Elmore, FEBS Letters 1 (1968) 343.
[7] H. Fritz, I. Eckert and E. Werle, Z. physiol. Chem. 348 (1967) 1120.
[8] F. Fiedler and E Werle, Z. physiol. Chem. 348 (1967) 1087.
[9] F. Fiedler, B. Müller and E. Werle, Z. physiol. Chem. 352 (1971) 1463.
[10] F. Fiedler and E. Werle, European J. Biochem. 7 (1968) 27.
[11] M.L. Bender and F.J. Kézdy, Ann. Rev. Biochem. 34 (1965) 49.
[12] M.L. Bender, F.J. Kézdy and F.C. Wedler, J. Chem. Education 44 (1967) 84.
[13] M.L. Bender, M.L. Begué-Cantón, R.L. Blakeley, L.J. Brubacher, J. Feder, C.R. Gunter, F.J. Kézdy, J.V. Killheffer, Jr., T.M. Marshall, C.G. Miller, R.W. Roeske and J.K. Stoops, J. Amer. Chem. Soc. 88 (1966) 5890.
[14] F.J. Kézdy and M.L. Bender, Biochemistry 1 (1962) 1097.
[15] H. Fritz, I. Trautschold and E. Werle, Z. physiol. Chem. 342 (1965) 253.
[16] T. Takami, J. Jap. Biochem. Soc. (Seikagabu) 41 (1969) 777.
[17] P. Andrews, Methods of Biochem. Analysis 18 (1970) 1.
[18] E. Habermann, Z. physiol. Chem. 328 (1962) 15.
[19] H. Moriya, J. Pharmacol. Soc. Japan (Yakugaku zasshi) 79 (1959) 1451.
[20] H. Moriya, A. Kato and H. Fukushima, Biochem. Pharmacol. 18 (1969) 549.
[21] E.J. Cohn and J.T. Edsall, Proteins, Amino Acids and Peptides (Reinhold Publishing Corp., New York, 1943) p. 370.
[22] H.K. Schachman, in: Methods in Enzymology, Vol. IV, eds. S.P. Colowick and N.O. Kaplan (Academic Press Inc., New York, 1957) p.32.
[23] R.A. Gibbons, in: Glycoproteins, ed. A. Gottschalk (Elsevier Publishing Comp., Amsterdam, London, New York, 1966) p. 29.
[24] T.E. Barman, Enzyme Handbook Vol. II (Springer-Verlag, Berlin, Heidelberg, New York, 1969).