THE REACTION OF PIG PANCREATIC KALLIKREIN WITH CINNAMOYL AND INDOLEACRYLOYL IMIDAZOLES

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1. Introduction

Acyl imidazoles like cinnamoyl imidazole react with serine proteinases, forming acyl enzymes with characteristic spectral properties [1–5]. Kallikrein (EC 3.4.4.21; the enzyme from porcine pancreas is understood throughout this paper) has recently been demonstrated to belong also to the group of pancreatic Asp–Ser–Gly-enzymes [6]. In the course of the present study we could show that kallikrein, too, forms a cinnamoyl and an indoleacryloyl enzyme. Spectral data of the acyl kallikreins and rate constants of their formation and hydrolysis have been determined. A comparison of these data with those of other serine proteinases reveals a rather close similarity of kallikreins A and B with bovine trypsin, though kallikrein is distinguished from trypsin e.g. by its carbohydrate content [7] and the outstanding specificity of its interactions with protein substrates and inhibitors [8].

2. Materials and methods

Trans-cinnamoyl imidazole was purchased from Cyclo Chemical Corp. and freshly recrystallized from cyclohexane. M.p. 132–133° (reported [9]: 133–133.5°). Indoleacryloyl imidazole was a gift from Dr. R.W.A. Oliver. Bovine trypsin (DCC treated, 1X cryst., lyophil., salt free) was obtained from Serva. Kallikrein was purified by the method of Fritz et al. [7] from prepurified enzyme preparations from pig pancreatic autolysates generously supplied by Farbenfabriken Bayer AG. It was treated with sialidase [7] and separated into the kallikreins A and B as previously described [10]. The preparations used had specific activities of 236 and 228 U/mg protein [6], respectively.

Solutions of kallikreins A or B (or, in some experiments, bovine trypsin) at the desired concentration (about 25 μM) were prepared in 0.1 M sodium acetate buffer, pH 5.2, containing 1 mM EDTA [11] and pressure filtered through a Sartorius membrane filter SM 11309 of 0.1 μm pore diameter. The concentration of enzymically active kallikrein was calculated from the absorbance using a redetermined value of $A^{1\%}_{280} = 20.5 \pm 0.5$ for the enzyme protein, a molecular weight of 24,000 for the protein moiety of kallikrein [12] and a specific activity of 300 U/mg protein for pure kallikrein [12]. Measurements were conducted on a Cary 15 recording spectrophotometer with 0–0.1 slide wire and 1 cm cuvettes thermostated at 25°. To 1.88 ml enzyme solution were added 20 μl of a 0.25 to 0.40 mM solution of the respective acyl imidazole in acetonitrile. Difference spectra against enzyme solution plus acetonitrile were recorded every 5 min. Alternatively, for a more precise determination of acylation rates, the decrease of indoleacryloyl (cinnamoyl) imidazole was followed at 410 (335) nm.

For the determination of the deacylation constants, about 1 hr after the start of the experiment 0.2 ml filtered 2 M Tris-HCl buffer (adjusted so as to give a final pH of 8.8) was added to each cuvette. The absorbance was recorded at 300 nm with cinnamoyl and at 355 nm with indoleacryloyl kallikrein.

For the determination of the rate of inactivation, 1.98 ml of a 0.25 μM solution of kallikrein A in the above acetate buffer was mixed with 20 μl of a 0.60 mM solution of cinnamoyl imidazole in acetonitrile.

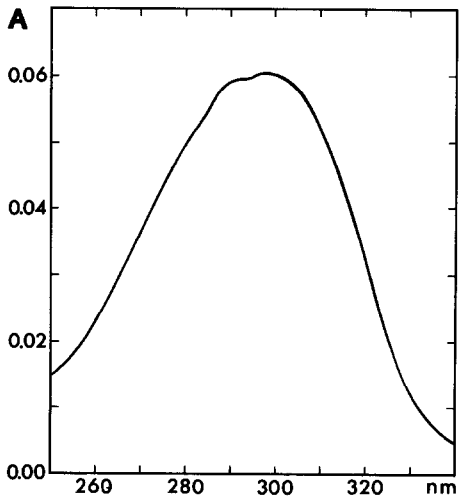

Fig. 1. Difference spectrum of trans-cinnamoyl kallikrein B against kallikrein B.

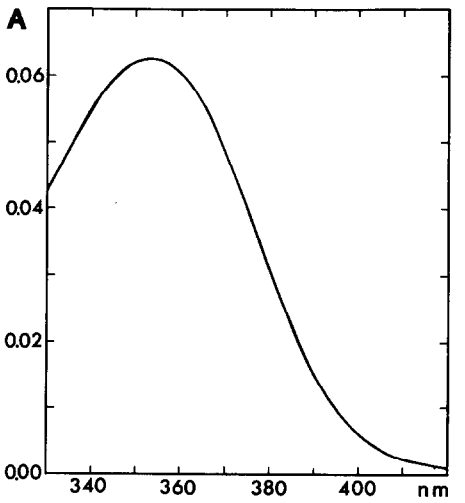

Fig. 2. Difference spectrum of indoleacryloyl kallikrein B against kallikrein B.

The mixture was kept at 25°, and samples of 50 to 200 µl were withdrawn every 3 min and introduced into cuvettes containing in a final volume of 3.0 ml 80 mM Tris-HCl buffer pH 8.0, 0.8 mM EDTA (11) and 0.5 mM α-N-benzoyl-L-arginine ethyl ester hydrochloride. The hydrolysis of the arginine ester was recorded at 25° for 1 min at 253 nm.

3. Results

About 15 min after the addition of cinnamoyl imidazole to a kallikrein solution, the difference spectrum against an identical solution of enzyme becomes essentially constant. Within the next 10 min, the absorbance at 298 nm decreases for less than 0.001 units. A typical difference spectrum is shown in fig. 1. In analogy with the previous work with other serine proteinases and based on the results with kallikrein presented below, the spectrum is interpreted as that of cinnamoyl kallikrein formed by acylation of a group in the active site of the enzyme. Fig.2 presents the corresponding difference spectrum of indoleacryloyl kallikrein which was recorded 25 min after the addition of substrate and did not change for the next 30 min. Spectra obtained with kallikrein A were identical to those of the corresponding acyl kallikreins B. Spectral data of the acyl kallikreins and our results with bovine trypsin are compiled in table 1, together with the respective data.

Indoleacryloyl imidazole is stable under our conditions, but cinnamoyl imidazole shows a slow spontaneous hydrolysis with rate constant $k_{hydr} = (1.39 \pm 0.024) \times 10^{-4} sec^{-1}$. This spontaneous hydrolysis was accounted for in the derivation of the rate equations used for the determination of k_{ac} (and k_{inh}), though the deviations from simple first order kinetics were not large. The derivations are straightforward and the equations are therefore not given here, but can be found in [13]. In order to simplify the kinetic treatment, a large excess of enzyme over substrate was desirable. Because of the high absorbance of the enzyme, however, only about an 8–10-fold excess was technically feasible. The condition $[E] \gg [S]$ is therefore satisfied only approximately, but the deviations expected are only small. Good straight lines resulted in semilogarithmic plots at least for two half lives with both acyl imidazoles.

The concomitant loss of the catalytic activity of kallikrein was followed in experiments conducted with a large excess of cinnamoyl imidazole until about 70% inhibition (at higher degrees of inhibition the deacylation reaction interferes with the activity determinations already in the 1 min measuring period). A rate constant k_{inh} of 119 ± 6 ($M^{-1} sec^{-1}$) was obtained for kallikrein A. This is identical within the

Table 1
Kinetic constants k_{ac} of the acylation of serine proteinases by cinnamoyl and indoleacryloyl imidazoles at pH 5.2, spectral data of the resulting acyl enzymes, and kinetic constants k_{deac} of their deacylation at pH 8.8.

	Cinnamoyl				Indoleacryloyl			
	k_{ac}	$k_{deac} \times 10^3$	λ_{max}	$\epsilon_{max} \times 10^{-4}$	k_{ac}	$k_{deac} \times 10^3$	λ_{max}	$\epsilon_{max} \times 10^{-4}$
	($M^{-1}sec^{-1}$)	(sec^{-1})	(nm)	($M^{-1}cm^{-1}$)	($M^{-1}cm^{-1}$)	(sec^{-1})	(nm)	($M^{-1}cm^{-1}$)
Kallikrein A	130 ± 8	3.4	298	1.80	260	1.2	353	2.24
Kallikrein B	116 ± 2	3.5	298	1.80	230	1.1	353	2.17
Trypsin			296	1.75			350	2.15
	63.4 [2]	17 [14]	296 [2]	1.93 [2]		3.6 [14]	350 [5]	(1.70) [5]
	46* [15]	15 [15]	296 [15]	1.8 [15]				
Chymotrypsin	12000 [2]	12.5 [14]	292 [1]	1.77 [1]	7000* [3]	1.9 [14]	359 [3]	2.00 [3]
			292 [14]	1.70 [14]			360 [4]	1.78 [4]
			292 [4]	1.78 [4]				
Subtilisin, Novo			289 [5]	1.89 [5]		3.8* [5]	358*[3]	2.00 [3]
							349 [5]	2.53 [5]
Subtilisin, Carlsberg			290 [5]	1.89 [5]		22* [5]	350 [5]	2.48 [5]

*Recalculated or taken from figures of the cited references.

experimental limits to k_{ac} derived from the spectrophotometric studies with a large excess of enzyme.

At pH 8.8, a fast decrease of absorbance of the acyl kallikreins was observed. Semilogarithmic plots showed good straight lines for more than two half lives. An identical k_{deac} for cinnamoyl kallikrein B was obtained by monitoring the formation of cinnamate ion by the increase of absorbance at 250 nm.

4. Discussion

Cinnamoyl and indoleacryloyl residues represent highly sensitive probes for the mode of binding and the environment of the chromophoric acyl group. The present study demonstrates that kallikrein is able to form acyl enzymes of the same general type as other serine proteinases. Since kallikreins A and B behaved identically in every respect within the experimental limits, the two forms of the enzyme are evidently very similar. While ϵ_{max} values of the different cinnamoyl and indoleacryloyl enzymes studies so far are not very different and rates of their hydrolysis are generally within an order of magnitude, the acylations of chymotrypsin proceed much faster than those of other enzymes. The velocity of acylation of bovine trypsin by cinnamoyl imidazole, however, is quite close to the value determined for kallikrein, especially if one considers that commercial trypsin preparations contain only 50–70% of active enzyme [16] so that the true rate constant for trypsin will be higher than the values given in table 1 by a factor of up to two. Comparison of the λ_{max} values of the different acyl enzymes shows that the acyl kallikrein spectra, too, resemble most closely those of the corresponding acyl trypsins, even as regards the fine structure of the cinnamoyl enzyme spectra [5]. From these results, a quite similar architecture of the active sites of kallikrein and of trypsin in the vicinity of the acyl probes is inferred. The narrow specificity of kallikrein in interactions with proteins [8] appears to have its structural basis outside this part of the enzyme molecule.

Acknowledgements

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