Action of urokinase and trypsin on angiotensin, ACTH and the oxidized B chain of insulin

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N-terminal analysis of the products of hydrolysis of angiotensin, ACTH and the oxidized B chain of insulin after 4 h incubation with trypsin and urokinase reveals a great qualitative similarity in the action of the two enzymes. As expected, the rates of hydrolysis differ significantly and are much higher in the case of trypsin catalysis than in the case of urokinase catalysis. Unexpectedly, however, a decrease in the difference between the catalytic activity of the two enzymes, by increasing the number of Arg and Lys residues present in the substrate, has been observed.

Trypsin *Urokinase specificity* *HPLC enzyme kinetics* *Angiotensin* *ACTH* *Oxidized insulin B chain*

1. INTRODUCTION

The proteolytic enzyme urokinase (EC 3.4.21.31) has attracted attention for several reasons. It is important as a potential drug against thromboembolic diseases and as a potent marker of tumorogenicity. Moreover, it is a very specific enzyme, cleaving a single Arg-Val bond in its natural substrate plasminogen. As yet no other natural substrate has been described for this enzyme. The reason for this high specificity is only poorly understood, although several small series of tripeptide substrates and inhibitors [1–4] have been studied in an attempt to estimate the importance of the amino acid residues adjacent to the scissile bond for the high values of the second-order rate constant ($k_{cat}/K_m = 2 \times 10^5\ M^{-1} \cdot s^{-1}$) for the activation of plasminogen by urokinase [5]. A very interesting observation was made by Sottrup-Jensen et al. [6], who reported that urokinase did not hydrolyze a 38-residue carboxymethylated fragment of plasminogen encompassing the region cleaved by urokinase in the activation of plasminogen.

The aim of this work was to study the action of urokinase towards 3 oligopeptide substrates. Parallel experiments with trypsin were carried out as well since urokinase is a trypsin-like enzyme. Angiotensin was chosen as a substrate because it has an Arg-Val bond, i.e. the bond that is cleaved in plasminogen; ACTH, because it has 3 Arg and 4 Lys residues; and the oxidized B-chain of insulin is a classical tool for studying proteinase specificity.

Abbreviations: DPCC, diphenyl carbamyl chloride; ACTH, adrenocorticotropic hormone, sequence 1–24; TEA-B, triethanolamine bicarbonate; HPLC, high-performance liquid chromatography; CTA, Committee of thrombolytic agents

2. MATERIALS AND METHODS

DPCC-treated bovine trypsin was purchased from Sigma. The concentration of the enzyme was determined by active-site titration with *p*-nitrophenyl-*p'*-guanidinobenzoate [7]. Urokinase was from Choay (Paris). Its active concentration was determined by an amidase test [8] assuming as in [9] 1 mol = 10×10^{12} CTA units. The preparation was considered free of other trypsin-like enzymes since the enzyme activity was not inhibited

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by aprotinin. Angiotensin II 5-Ile and ACTH seq. 1–24 p.A. were from Serva. The oxidized B chain of bovine insulin was from Sigma. All substances used for buffer preparation were reagent grade and CH_3CN of HPLC grade was from Fluka.

The bonds cleaved by the enzymes after 4 and 6 h incubation at 37°C, pH 8.5, in 0.2 M TEA-B buffer, at a substrate-enzyme molar ratio of 100:1, using 100 nmol peptide (3×10^{-4} M) were determined by identification of the N-terminal residues by the dansyl chloride method [10] with separation of the dansylamino acids on polyamide sheets [11]. The rate of hydrolysis of the substrates was followed by HPLC: RP-18μ Bondapack analytical column, Waters solvent delivery system 6000 A, U6K universal injector, 441 absorbance detector, monitoring at 214 nm. The rate of disappearance of the substrates was measured under pseudo-first-order conditions ($K_m \gg S_0 > E_0$) by following the decrease of the substrates' peak surfaces. The experimental conditions are summarized in table 1. In all cases the hydrolysis was carried out at 37°C in 0.2 M TEA-B buffer, pH 8.5, 0.1 M NaCl. At appropriate time intervals 5–10 μl aliquots were withdrawn from the reaction mixture and injected into the chromatograph.

3. RESULTS AND DISCUSSION

According to the N-terminal analysis, the bonds cleaved in the 3 substrates by urokinase and trypsin can be schematically represented as follows (arrows on the upper side of the peptide chain are for trypsin and those below, for urokinase):

Angiotensin: Asp-Arg↓↑Val-Tyr-Ile-His-Pro-Phe

N-terminal Asp and Val were found both after trypsin and urokinase action.

Oxidized B chain of insulin: Phe-Val-Asn-Glu-His-Leu-(SO$_3$H)Cys-Gly-Ser-His-Leu-Val-Gly-Ala-Leu-Tyr-Leu-Val-Val-(SO$_3$H)Cys-Gly-Glu-Arg_{22}↓↑Gly_{23}-Phe-Phe-Tyr-Thr-Pro-Lys_{29}↓↑Ala_{30}

In the case of trypsin N-terminal Phe, Gly and Ala were detected after 4 h hydrolysis. In the case of urokinase only Phe and Ala appeared after 4 h hydrolysis. N-terminal Gly appeared only after 6 h hydrolysis. This indicates that the Lys_{29}-Ala_{30} bond is split at a faster rate than the Arg_{22}-Gly_{23} bond by urokinase, whereas in the case of trypsin the reverse is true [12].

ACTH: Ser-Tyr-Ser-Met-Glu-His-Phe-Arg↓-Trp-Gly-Lys↓-Pro-Val-Gly-Lys_{15}↓↑Lys_{16}↓↑Arg_{17}↓↑Arg_{18}-↓-Pro_{19}-Val-Lys↓↑Val-Tyr-Pro

N-terminal Ser, Lys, Arg and Val were detected after urokinase hydrolysis and the same as well as Pro, after trypsin hydrolysis. Since Trp is decom-

Table 1

Conditions for enzyme hydrolysis of the substrates and HPLC analysis

Substrate	Isocratic elution with	Flow rate (ml/min)	Retention time (min)	E_0 (M)	E_0/S_0 mole ratio
Angio-tensin	0.067 M KH_2PO_4-H_3PO_4, pH 2.1, 20% CH_3CN (v/v)	2	6	T 5×10^{-7} U 6×10^{-6}	1:340 1: 28
B chain	0.067 M KH_2PO_4-H_3PO_4, pH 2.1, 30% CH_3CN	2	6.5	T 2×10^{-7} U 2×10^{-6}	1:200 1: 20
ACTH	0.067 M KH_2PO_4-H_3PO_4, pH 2.2, 20% CH_3CN	1.5	5	T 2×10^{-7} U 2×10^{-6}	1:815 1: 80

T, trypsin; U, urokinase

posed during the 20 h HCl hydrolysis, it could not be detected as N-terminal. The arrow for trypsin at Arg-Trp is from literature data [13].

The sequence Lys_{15}-...-Arg_{18} makes it difficult to decide which of the bonds split produce the N-terminal Arg found. Literature data for trypsin [13] show that splitting occurs at all these bonds as shown by the arrows. The results from the HPLC experiments (using monitoring at 214 nm, free Lys cannot be detected at the substrate concentrations used, while Arg can) show that free Arg is present both in the trypsin and the urokinase hydrolysates of ACTH. This indicates that splitting at a measurable rate by urokinase occurs at both Lys_{16}-Arg_{17} and Arg_{17}-Arg_{18}.

It is noteworthy that N-terminal Pro has not been mentioned in previous studies of trypsin action on ACTH [13]. The reason for this should be the greater sensitivity of the dansyl chloride method over the one used earlier. The presence of an Arg-Arg peak, observed during the HPLC experiments, is a proof that the Arg_{18}-Pro_{19} is cleaved. The dotted arrow at Lys_{11}-Pro_{12} is to show that it is not certain whether this bond is also split by trypsin.

The results from the N-terminal analysis show a great although qualitative similarity between trypsin and urokinase. To compare the enzymes quantitatively, the rate of decrease of the substrates due to enzyme hydrolysis was followed by HPLC. A linearity of ln S_0/S with time till at least 50% conversion of the substrates was observed. From the slopes, the pseudo-first-order rate constants (k_I) and k_I/E_0, which is a measure of specificity, were calculated and are shown in table 2.

In the case of the B chain the elution conditions were such that des-Ala B was not separated from B and the decrease of the B-peak was due only to the cleavage of the Arg_{22}-Gly_{23} bond. Thus, a comparison between the rate of disappearance of the B chain and of angiotensin is a comparison between the rate of Arg-Val and Arg-Gly bond breaking. The value of k_I/E_0 for urokinase is 26-times higher for the hydrolysis of the B chain than for angiotensin. On both sides of the scissile bond of the 2 substrates the amino acid residues are similar: Glu and Phe in the B chain, Asp and Tyr, respectively, in angiotensin. Val should be a better 'leaving group' since an Arg-Val bond is cleaved in plasminogen by urokinase. The higher rate of Arg-Gly bond breaking than of Arg-Val indicates that it is not the adjacent amino acid residues that are important for urokinase catalysis.

Another result which is noteworthy, is that while in the case of urokinase the k_I/E_0 value increases by 2 orders of magnitude from angiotensin to ACTH, in the case of trypsin the respective k_I/E_0 value increases only about twice (table 2). The ratio between k_I/E_0 for trypsin and for urokinase in the case of angiotensin hydrolysis is 791 and in the case of ACTH hydrolysis, only 24. It thus appears that with increasing number of Arg and Lys residues in a substrate, the difference between the 2 enzymes decreases and one could expect that urokinase would not behave as a very specific enzyme towards high molecular mass substrates.

Table 2

Kinetic parameters for the hydrolysis of the substrates by trypsin (T) and urokinase (U)

Substrate	k_I (min^{-1})	k_I/E_0 ($M^{-1} \cdot s^{-1}$)	$\frac{k_I/E_0\ (T)}{k_I/E_0\ (U)}$
Angiotensin	T $(3.3 \pm 0.3) \times 10^{-2}$	1100	791
	U $(5 \pm 0.5) \times 10^{-4}$	1.39	
B chain	T $(4.9 \pm 0.6) \times 10^{-2}$	4083	112
	U $(4.3 \pm 0.5) \times 10^{-3}$	36.25	
ACTH	T $(5.8 \pm 0.7) \times 10^{-2}$	4833	24
	U $(2.4 \pm 0.4) \times 10^{-2}$	200	

However, only one out of the 41 Arg(Lys)-X bonds in plasminogen is cleaved by urokinase. All this strongly suggests that the reason for the high specificity of urokinase in activating plasminogen should be looked for in the overall tertiary structure and conformation of plasminogen itself, rather than in the importance of the amino acid residues in close proximity to the scissile bond.

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