Biochimica et Biophysica Acta, 429 (1976) 252—257

BBA 67723

IDENTIFICATION OF AN ACTIVE SITE HISTIDINE IN UROKINASE

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(Received August 1st, 1975)

Summary

Two forms of urokinase (EC 3.4.99.26) with apparent molecular weights of 33 400 and 47 000 purified by affinity chromatography have been modified specifically with newly synthesized peptide chloroketones by affinity labeling. Rapid inactivation of the enzyme preparations was observed with Ac-Gly-Lys-CH_2 Cl and Nle-Gly-Lys-CH_2 Cl which might be associated with a change in which a histidine residue is lost. After performic acid oxidation, an equivalent amount of 3-carboxymethyl histidine could be recovered, indicating alkylation at the N-3 of a histidine residue. In the case of the norleucine derivative, norleucine was concomitantly incorporated into the protein. It is thus likely that urokinase belongs in the class of enzymes utilizing the Asp..His..Ser triad for their catalytic action. The two active site residues so far identified, serine and histidine, were located in the heavy chain (33 100 mol. wt) of the 47 000 molecular weight form and in the 33 400 molecular weight form, the molecular weight of which remained constant.

Introduction

Urokinase (EC 3.4.99.26), a proteolytic enzyme with trypsin-like specificity for esters of synthetic substrates, also catalyzes the conversion of plasminogen to plasmin, which then participates in fibrinolysis of thrombi during wound healing of the genito-urinary tract in man. Urokinase has also been administered intravenously as a therapeutic agent for thromboembolic disease in man [1,2]. A plasminogen activator which is cellular in origin is associated with oncogenesis [3].

Several reports have appeared on the isolation, purification and/or characterization of urokinase. Lesuk et al. [4] crystallized urokinase and proposed a molecular weight of 54 000 but others have described urokinase preparations

of 31 000 to 54 000. In our laboratory, a 33 400 and a 47 000 molecular weight form of urokinase were purified by affinity chromatography to homogeneity on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. On reduction, the 47 000 molecular weight fraction was made up of a heavy chain of 33 100 and a light chain of 18 600 whereas the 33 400 molecular weight fraction did not change (Soberano, M.E., Ong, E.B., Johnson, A.J., Schoellmann, G. and Levy, M., unpublished).

Urokinase proved sensitive to diisopropylphosphorofluoridate (DFP) and the active site titrant (inhibitor) *p*-nitrophenyl-*p'*-guanidinobenzoate, presumably by reacting with an essential serine residue [5,6]. Soberano et al. [5] incorporated ^{3}H-labeled DFP and ^{14}C-labeled *p*-nitrophenyl-*p'*-guanidinobenzoate into the urokinase and demonstrated that the active site serine residue is located in the 33 100 molecular weight heavy chain.

Chloroketones derived from various amino acids and peptides were also shown to be active site-directed inhibitors for various enzyme systems [7—9].

The present communication concerns an active site histidine residue identified in urokinase through the use of newly synthesized, relatively specific, peptide chloroketones, acetylglycyl-L-lysyl chloromethane, and L-norleucyl-glycyl-L-lysyl chloromethane.

Materials and Methods

Twice crystallized, salt-free, lyophilized trypsin (Code TRL) was obtained from Worthington; human urokinase (Lots 48 and 54 with a specific activity of 75 000, and 92 000 CTA units/mg protein) was procured from Serono. Standardized urokinase, prepared for the World Health Organization (4800 CTA units/ampule), was used as a control to determine the enzyme activity. All other reagents and chemicals were of the highest grade available.

The lysine chloroketone Lys-CH_2 Cl was synthesized essentially according to the method of Shaw and Glover [10] from dicarbobenzoxylysine, except that the diazoketone intermediate was prepared by the mixed anhydride method in which the acid is activated with ethyl chloroformate. The end product, obtained in good yield, had a melting point of 172—174°C (Lit. m.p. 170—172°C). The hydrochlorides of Ac-Gly-Lys-CH_2 Cl and Nle-Gly-Lys-CH_2 Cl were synthesized from the common starting material *N*-ε-Cbz-*N*-α-Boc-Lys. The intermediate diazoketone was prepared by activating the acid with ethyl chloroformate then converting to the fully protected lysine chloroketone. After removal of the *t*-Boc-group, Ac-Gly and Cbz-Nle-Gly were each coupled to the liberated α-amino groups, again by the mixed anhydride procedure. After removal of the protecting groups the desired peptide chloroketones were obtained. A more detailed account of the synthesis of these compounds will be published elsewhere.

The two forms of urokinase (47 000 and 33 400 mol. wt) were isolated and purified on a Sepharose-ε-aminocaproyl-agmatine column by the method of Soberano et al. [5].

Urokinase activity was estimated by the protamine assay using a Technicon Auto Analyzer II [11]. The assay was routinely calibrated against urokinase and leucine standards. Urokinase concentrations were measured by titration with

p-nitrophenyl-*p'*-guanidinobenzoate by a modification of the method of Chase and Shaw [12]. Urokinase concentrations were also determined spectrophotometrically at 280 nm based on an absorbance of 12.6 for a 1% solution. Trypsin activity was assayed with Tos-Arg-OMe as the substrate, by Hummel's spectrophotometric method [13].

Control studies were performed with the new chloroketone inhibitors using trypsin as a typical serine protease. The trypsin was rapidly inhibited in 0.1 M Tris/0.02 M $CaCl_2$ buffer (pH 7.4) at room temperature, by both peptide chloroketones, confirming their effective reaction with the enzyme's active site. Structural studies performed on the active site labeled trypsin showed stoichiometric incorporation of the peptide moiety and the alkylation site was identified as the N-3 of the active site histidine residue.

In studying urokinase, the enzyme was incubated with the chloroketones in 0.06 M *N*-ethylmorpholine/0.09 M NaCl (pH 7.5) at 37° C, and samples were withdrawn at various intervals over a 60-min period for activity measurements. After incubation, some of the samples were dialyzed for at least 24 h against several changes of the buffer, then examined for activity. Samples of urokinase and its derivatives which were intended for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and amino acid analysis were prepared identically except that 0.15 M *N*-ethylmorpholine was substituted for the dialysis buffer. The dialyzed preparations were freeze-dried and kept until needed.

Amino acid analyses were carried out with a Jeol amino acid analyzer equipped with an Autolab System AA computing integrator, or on a Beckman Spinco Analyzer, Model 120 C, equipped with a manual sample injector. The protein samples were hydrolyzed with 5.7 M HCl in sealed, evacuated ignition tubes at 110° C for 22 h. If performic acid oxidation preceded hydrolysis, Hirs' method [14] was used. Hydrolysates of gel slices for amino acid analysis were prepared as described by Houston [15] except that 1.0 ml of 0.2 M NaOH was added to each of the dried hydrolysates. They were evaporated to near dryness to remove excess ammonia, then acidified with 1.0 ml of 1 M HCl. After the samples were thoroughly dried, each was reconstituted with 0.9 ml of 0.01 M HCl.

A modified Weber and Osborn sodium dodecyl sulfate-polyacrylamide gel electrophoresis [16] was performed. After electrophoresis and staining with Coomassie brilliant blue, the amino acid composition of the major bands was determined on the acid-hydrolyzed samples.

Results and Discussion

The design of the active site-directed inhibitors newly synthesized for use with urokinase was based on the effectiveness of Ac-Lys-OMe and Ac-Gly-Lys-OMe as synthetic substrates for urokinase [17]. The results of inactivation studies with some of the chloromethyl ketone inhibitors are summarized in Fig. 1. Although Tos-Lys-CH_2 Cl is a poor inhibitor of urokinase, it was included at the relatively high concentration of 54.0 mM for purposes of comparison and showed definite evidence of inhibition, i.e. 60% after 1 h of incubation at pH 7.5. The data of Landmann and Markwardt [6] did not show such an inhibitory effect, a circumstance which may be explained by their use of

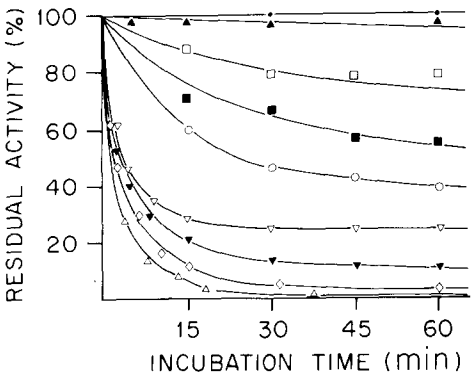

Fig. 1. Inactivation of urokinase by chloromethyl ketones. Unless stated otherwise, urokinase (54 μM) was incubated with the chloromethyl ketones in 0.06 M *N*-ethylmorpholine/0.09 M NaCl, pH 7.5, at 37° C. Reaction of the enzyme with various materials is shown by the symbols following each: No inhibitor added, ●———●; Tos-Lys-CH_2Cl (1.2 mM) at 20° C, ▲———▲; Lys-CH_2Cl (54 mM), pH 7.0, □———□; Tos-Lys-CH_2Cl (54 mM), pH 7.0, ■———■; Tos-Lys-CH_2Cl (54 mM) ○———○; Ac-Gly-Lys-CH_2Cl (159 μM), ▽———▽; Nle-Gly-Lys-CH_2Cl (159 μM), ▼———▼; Nle-Gly-Lys-CH_2Cl (222 μM) and urokinase (43.1 μM), ◇———◇; Ac-Gly-Lys-CH_2Cl (270 μM), △———△.

low concentrations of both enzyme and inhibitor, conditions under which we also observed only marginal inactivation (1.2 mM chloroketone).

Tos-Lys-CH_2 Cl was more effective than Lys-CH_2 Cl at pH 7.0. But the peptide chloroketones were much better inhibitors of the enzyme than the Tos-Lys-CH_2 Cl when incubated at pH 7.5. When urokinase was incubated with as little as a 3-fold excess of the dipeptide chloroketone the enzymic activity was rapidly lost. From these data, it would seem the subsite interaction is important in the interaction with this enzyme, as noted in numerous other cases.

The tripeptide chloroketone Nle-Gly-Lys-CH_2 Cl was synthesized with the added norleucine residue as a marker for the structural studies with modified urokinase and proved even more effective than the dipeptide chloroketone, probably indicating still more extended subsite interaction with the urokinase. It is interesting that the non-physiologic amino acid norleucine does not inhibit formation of the urokinase-chloroketone complex, and may even contribute to it.

Reaction of urokinase with the chloromethyl ketones showed that the enzyme activity (monitored by the protamine assay) and the concentration of urokinase (titrated with *p*-nitrophenyl-*p'*-guanidinobenzoate) were affected to about the same degree. When the inhibited enzyme was assayed again after overnight dialysis with several changes of buffer, no enzymic activity was regained, confirming the relatively irreversible nature of the reaction and the strength of the enzyme-inhibitor bond.

Direct evidence for an active center histidine residue was obtained initially with only partially purified urokinase. Amino acid analysis of this material after alkylation revealed a definite loss of histidine and, after performic acid oxidation, recovery of 3-carboxymethyl-histidine. These findings indicated that the N-3 of a histidine residue was alkylated by the inhibitors. With use of the

TABLE 1

AMINO ACID ANALYSIS OF UROKINASE AND Nle-Gly-Lys-CH_2Cl-TREATED UROKINASE
The values are the average of four analyses.

Urokinase preparation	Amino acid residue		
	Histidine	3-Carboxymethyl histidine	Norleucine
33400 molecular weight form*			
Urokinase alone	9.6	0	0
Nle-Gly-Lys-CH_2Cl-treated urokinase	8.9	0	0.9
Urokinase alone, performic acid oxidized	9.6	0	0
Nle-Gly-Lys-CH_2Cl-treated urokinase, performic acid oxidized	8.7	0.5	0.8
47000 molecular weight form**			
Urokinase alone	17.0	0	0
Nle-Gly-Lys-CH_2Cl-treated urokinase	16.4	0	4.3 ***
Urokinase alone, performic acid oxidized	17.0	0	0
Nle-Gly-Lys-CH_2Cl-treated urokinase, performic acid oxidized	16.2	0.6	3.9***

* Calculations were based on 33 residues of glutamic acid.

** Calculations were based on 13 residues of phenylalanine.

*** The histidine loss alone cannot account for this high value for the 47 000 molecular weight urokinase. The reasons, other than the influence of histidine modification, are not yet clear, and efforts are being made to resolve the problem. Nonetheless, the data do not nullify the histidine results as reflected by the recovery of 3-carboxymethyl histidine and the reduction in histidine after reaction with the inhibitor.

peptide chloroketone containing norleucine, incorporation of the norleucine into the protein samples was easily demonstrated by amino acid analysis.

When each of the highly purified 47 000 and 33 400 molecular weight forms of urokinase, obtained by affinity chromatography, was reacted with the tripeptide chloroketone: (a) a histidine residue in the active center of the urokinase was modified at the N-3 position and (b) the norleucine was concomitantly incorporated into the protein (Table I). In addition, (c) the norleucine was bound to the 47 000 and 33 400 molecular weight bands after sodium dodecyl sulfate-polyacrylamide gel electrophoresis; and, after reduction of each form with dithiothreitol, (d) the norleucine was found to be incorporated into the heavy chain (mol. wt 33 100) of the 47 000 molecular weight form and into the 33 400 form whose molecular weight remained constant.

Since a serine residue in urokinase has been identified previously [5,6], this enzyme can be added to the class of active site serine enzymes which depend upon the Asp..His..Ser triad for their catalytic action. Both amino acid residues have now been identified in the 33 400 molecular weight form as well as the heavy chain (33 100 mol. wt) of the 47 000 molecular weight form of urokinase. Structural studies of these enzyme derivatives are under way in our laboratories.

Acknowledgments

The authors wish to thank Mrs. E. Dharmgrongartama for expert technical assistance; Dr. M.E. Soberano for performing the acrylamide gel electrophoresis; and Mrs. E.T. Wadsworth for editorial help. This work was supported in part by U.S.P.H.S. grant HL-15596.

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