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KRINGLE 5 OF HUMAN PLASMINOGEN CARRIES A BENZAMIDINE-BINDING SITE

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SUMMARY: Affinity of plasminogen fragments for p-aminobenzamidine-Sepharose was investigated to localize the benzamidine-binding site(s) of the protein. i/ Of the elastase fragments of plasminogen only miniplasminogen (kringle 5 plus light chain) was bound to the column. Kringle 1+2+3 and kringle 4, which carry the lysine-binding sites, were not adsorbed, proving that the lysine- and benzamidine-binding sites are on different domains of the protein. ii/ Light chain was bound to the column even if the primary benzamidine-binding site was covalently blocked, indicating that the protease part of plasmin has a second benzamidine-binding site. iii/ Kringle 5 also binds to the affinity column: the presence of a binding site on kringle 5 raises the possibility that this structure may take part in the interactions of plasminogen with other proteins.

Plasmin, a serine protease with a broad, trypsin-like specificity is the key enzyme of the fibrinolytic system. Both plasmin and its inactive proenzyme, plasminogen have the ability to form complexes with several other components of the fibrinolytic system, such as fibrin, α_2-antiplasmin and plasminogen activators. Wiman and Collen formulated a unified model of fibrinolysis which assumes that these protein-protein interactions are crucial for the regulation of plasmin action and are responsible for restricting its proteolytic activity to fibrin in vivo (1). These protein-protein interactions are probably realised through multiple-point connections between well defined structures of the complementary surfaces of the interacting proteins. Five closely homologous triple-loop structures (kringles) are present in the heavy chain, non-protease part of the molecule (2), and these kringle structures have been implicated in harbouring the binding sites for α_2-antiplasmin (3) and fibrin (4).

Plasminogen is also capable of binding low Mw compounds. The binding of ω-aminocarboxylic acids to plasminogen has been studied extensively (5-9),

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and it has been shown that the ω-aminocarboxylic acid binding sites (so-called lysine-binding sites) are located on kringles (2) and are closely related to the sites interacting with fibrin (4) and α_2-antiplasmin (3).

Much less is known about the location of the sites responsible for the affinity of plasminogen for benzamidine-type compounds. Holleman et al. (10) have shown previously that plasminogen can bind to p-aminobenzamidine subsituted Sepharose and provided evidence that the binding sites for ω-aminocarboxylic acids and benzamidine are independent since lysine can not elute plasminogen from p-aminobenzamidine-Sepharose and vice versa. Markwardt et al. have reported that benzamidines are potent competitive inhibitors of plasmin when either the amidase or the fibrinolytic activity of the enzyme is assayed and suggested that these compounds are bound in the specificity pocket of the enzyme (11), in analogy with the binding of these inhibitors to trypsin (12) and other trypsin-like proteases (13). However the benzamidine-binding site studied by Holleman et al. is not identical with this primary specificity site of the enzyme as plasmin inactivated either with p-nitrophenyl-p'-guanidinobenzoate or p-(m(m-fluorosulfonylphenylureido)-phenoxyethoxy) benzamidine showed affinity for p-aminobenzamidine-Sepharose (10).

Neither the location nor the number of the benzamidine-binding sites of plasminogen is known. In the present paper we localize two benzamidine-binding sites in addition to the one identical with the primary specificity site of human plasmin.

MATERIALS AND METHODS

p-Aminobenzamidine (Serva), 1-ethyl-3-(3 dimethylaminopropyl)-carbodiimide (Fluka), benzamidine (Merck), p-nitrophenyl-p'-guanidinobenzoate (Polyscience), CH-Sepharose 4B (Pharmacia) and streptokinase (Kabi) were commercial products. Porcine pancreatic elastase purified according to Shotton (14) was a generous gift of Dr. P. Tolnay (Institute of Drug Research, Budapest).

Human native plasminogen (Glu-plasminogen) was prepared from fresh citrated plasma by affinity chromatography on lysine-Sepharose as described by Deutsch and Mertz (15), the purification steps were performed in the presence

of bovine pancreatic trypsin inhibitor (Trasylol, Bayer) to prevent conversion of Glu-plasminogen to Lys-plasminogen. The purity of isolated plasminogens was checked routinely by polyacrylamide gel-electrophoresis in urea/acetic acid, pH 3.2, as described by Walther et al. (16).

Plasminogen was digested with porcine pancreatic elastase and the fragments, kringle 1+2+3, kringle 4 and miniplasminogen were subsequently purified by affinity chromatography on lysine-Sepharose and gel-filtration on Sephadex G-75 essentialy as described by Sottrup Jensen et al. (2). The purity of the isolated fragments was checked with 6-16% linear polyacrylamide gradient SDS slab gel-electrophoresis under both reducing and non-reducing conditions.

Activation of miniplasminogen, preparation of guanidinobenzoyl derivative of miniplasmin and the selective reduction and alkylation of the inter-chain disulphide bridges was performed by the method described for plasminogen by Wiman (17).

p-Aminobenzamidine was coupled to CH-Sepharose 4B by 1-ethyl-3-(dimethyl-aminopropyl)-carbodiimide as described by Schmer (18).

Affinity of plasminogen and plasminogen fragments for p-aminobenzamidine-Sepharose was assayed at 25°C by column chromatography. In the assay the samples never exhausted more than 20% of the column-capacity. The column (0.6 x 5 cm) was equilibrated with 0.1 M ammonium bicarbonate, pH 8.0. After loading the sample onto the column, it was washed with five bed-volumes of 0.1 M ammonium bicarbonate, pH 8.0 to remove proteins devoid of affinity. The bound proteins were eluted either with buffer containing 0.2 M benzamidine or a linear gradient of benzamidine using 15 ml of buffer in the mixing chamber and 15 ml of buffer containing 0.15 M benzamidine in the reservoir and elution was completed with five bed-volumes of buffer containing 0.15 M benzamidine.The benzamidine containing fractions were desalted by gel-filtration on a Sephadex G-25 column equilibrated with 0.1 M ammonium bicarbonate, pH 8.0.

Protein samples were hydrolysed for 24 hours in 6 N HCl at 110°C and the amino acid composition of the hydrolysates was determined on a JEOL JLC 5AHA analyzer.

RESULTS AND DISCUSSION

Plasminogen has been shown previously to bind to p-aminobenzamidine-Sepharose (10). In the present study we confirmed this finding and identified the regions of the protein which carry benzamidine-binding sites.

By limited proteolysis of plasminogen with porcine pancreatic elastase three nonoverlapping fragments can be obtained: kringle 1+2+3 (Tyr_{79}-Val_{337}, Tyr_{79}-Val_{353}), kringle 4 (Val_{354}-Ala_{439}) and miniplasminogen (Val_{442}-Asn_{796})(2). Kringle 1+2+3 and kringle 4, the fragments with lysine binding sites, failed to adsorb to p-aminobenzamidine-Sepharose. Only miniplasminogen, the plasminogen fragment which lacks a lysine-binding site, adsorbed to p-aminobenzamidine-Sepharose and could be eluted from the column with 0.2 M benzamidine in 0.1 M ammonium bicarbonate buffer, pH 8.0. Miniplasminogen is, however, not eluted

from the affinity column with o.3 M ammonium bicarbonate buffer, pH 8.o (buffer with similar ionic strength as that of the benzamidine containing eluant), indicating that the elution is due to a ligand specific effect and the immobilized p-aminobenzamidine binds miniplasminogen probably via a benzamidine binding site.

We can thus conclude that lysine-binding sites and benzamidine-binding site(s) are not only independent, but they are located on different domains of plasminogen. The benzamidine-binding site(s) reside in the miniplasminogen region which consists of the fifth kringle and the serine protease segment of plasminogen.

Miniplasmin formed by activation of miniplasminogen with streptokinase was inactivated with p-nitrophenyl p'-guanidinobenzoate. This miniplasmin derivative showed affinity for p-aminobenzamidine-Sepharose in harmony with the conclusion of Holleman et al. (lo) that the primary specificity site of plasmin, expected to be occupied by the guanidinobenzoyl group of the reagent, can not account for the p-aminobenzamidine-Sepharose affinity.

To decide whether the light chain or the kringle 5 region carries the benzamidine-binding site(s), p-guanidinobenzoyl miniplasmin was subjected to selective reduction and alkylation and the resulting mixture of kringle 5 and p-guanidinobenzoyl light chain (containing also some unreduced p-guanidinobenzoyl miniplasmin) was applied onto the p-aminobenzamidine-Sepharose column. All three components of the sample remained bound to the column and were eluted with a linear benzamidine gradient as shown in Fig.1. The components eluted with benzamidine were identified by their relative mobilities in SDS gel-electrophoresis and their amino acid composition on the basis of the known sequence of plasminogen (2,17): residues Val_{442}-Arg_{56o} and Val_{561}-Asn_{79o} corresponding to kringle 5 and light chain, respectively.

The above experiments thus revealed that benzamidine binding sites are present both in kringle 5 and the light chain portion of miniplasminogen.

On the basis of the present findings there are at least three different

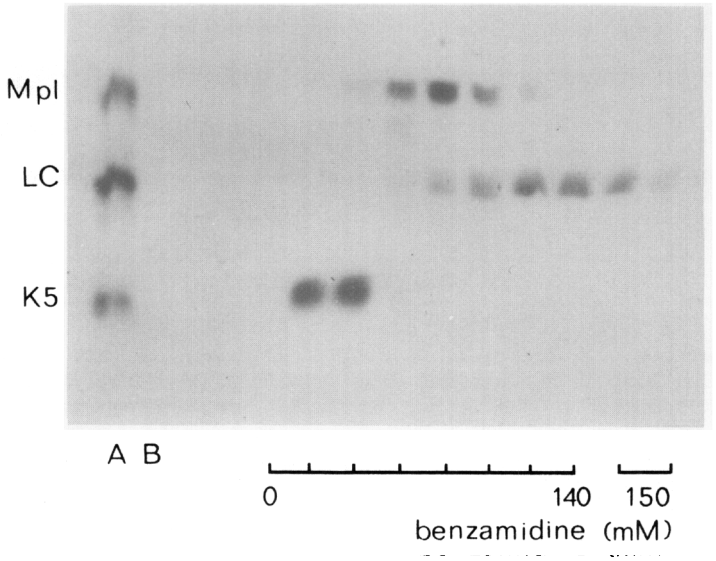

Fig.1. SDS polyacrylamide gel-electrophoretic pattern of fractions eluted from p-aminobenzamidine-Sepharose column with benzamidine gradient. The sample (A) contained p-guanidinobenzoyl miniplasmin (Mpl), p-guanidinobenzoyl light chain (LC) and kringle 5 (K5). The sample was applied onto a p-aminobenzamidine-Sepharose column (0.6 x 5 cm) and washed with five bed-volumes of 0.1 M ammonium bicarbonate, pH 8.0.(B). The bound components were eluted with a linear 0-150 mM benzamidine gradient and elution was completed with buffer containing 0.15 M benzamidine.

structures in human plasmin capable of binding benzamidine-type compounds. One of these sites is responsible for the primary specificity of plasmin which binds the basic side-chain adjacent to the scissile bond of the substrate (11). Since blocking of this site with active site titrants, such as p-nitrophenyl-p'-guanidinobenzoate, does not eliminate the affinity of light chain for the p-aminobenzamidine-Sepharose, this indicates that there is a second benzamidine-binding site in the catalytic chain. Studies on the inhibitory action of mono-, bis- and tris-benzamidines have suggested the presence, in several trypsin-like proteases, of a second benzamidine-binding site (13). Our present finding suggests that this is also true for plasmin.

Interestingly, a further benzamidine-binding site is carried by the kringle 5 portion. The kringle structures have been shown to be involved in binding of ω-aminocarboxylic acids, α_2-antiplasmin and fibrin to plasminogen. It seems likely that these unusual structures are essential as sites

for the multiple-point interactions with different proteins. Until now no binding site has been assigned to kringle 5. Our finding that kringle 5 possesses a binding site raises the possibility that this domain may also participate in the interactions of human plasminogen with other proteins.

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