C-terminal lysine residues of fibrinogen fragments essential for binding to plasminogen

Ulla Christensen

Chemical Laboratory IV, Universitetsparken 5, DK-2100 Copenhagen, Denmark

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Experiments involving affinity chromatography on immobilized plasminogen columns and the concomitant use of plasmin and carboxypeptidase B indicate that the COOH-terminal lysine residues formed by plasmin-catalyzed cleavage of fibrinogen are essential for the high-affinity binding of the resulting cleavage products to plasminogen.

C-terminal lysine residue *Fibrinogen* *Plasminogen* *Lysine binding site* *Fibrinogen fragment D* *Fibrinogen fragment E*

1. INTRODUCTION

The so-called lysine-binding sites of plasminogen and plasmin apparently are important sites in the regulation of fibrinolysis [1,2]. Distinction between two or three classes of lysine-binding sites, weak and strong sites, is made from their affinity to ω-aminocarboxylic acids analogous to lysine [3,4]. As recently noted [2], C-terminal lysine residues of proteins and peptides, as I see it, are the only likely compounds known to be present physiologically that fulfill exactly the apparent ligand structure requirements of the strong class of plasmin(ogen) lysine-binding sites. The effects mediated by strong lysine-binding sites in the regulation of fibrinolysis and perhaps other systems that involve plasmin(ogen) thus may depend on the presence of C-terminal lysine residues of the proteins. In particular, the progress of fibrinolysis may depend on the C-terminal lysine residues formed when plasmin cleaves fibrin, whereas the initiation of the process may depend on the AH-site of plasminogen, the weak lysine-binding site reported on in [2].

The results of this report strongly indicate that the C-terminal lysine residues of plasmin-cleaved fibrinogen fragments are essential for high-affinity binding of the fragments to immobilized plasminogen.

2. EXPERIMENTAL

2.1. *Reagents*

Human plasminogen (Lys) was prepared as in [2]. Plasminogen-substituted Sepharose 4B was prepared by coupling of plasminogen to 10 ml CNBr-activated Sepharose 4B (final concentration approx. 10 nmol/ml gel) using the procedure recommended by the manufacturer (Pharmacia, Uppsala). Human plasmin was prepared by conversion of plasminogen on a column of urokinase-substituted Sepharose 4B as described in [5].

The following materials were obtained from the indicated commercial sources: human fibrinogen, grade L and D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (S-2251) (Kabi, Stockholm); Apotinin (Trasylol®), (Bayer, Leverkusen, FRG); carboxypeptidase B from porcine pancreas (DFP-treated) (Boehringer, Mannheim); monospecific antibodies raised in rabbits against human fibrinogen (Dakopatts, Copenhagen), and against human fibrinogen degradation product D (anti-FDP D) and E (anti-FDP E) (Hoechst Danmark, Copenhagen); 6-aminohexanoic acid (Fluka, Buchs, Switzerland); urokinase (Leo, Copenhagen).

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2.2. *Samples*

After removal of plasminogen on lysine-substituted Sepharose 4B, 5 aliquots of a fibrinogen solution [each aliquot 5 ml, containing approx. 10 nmol (2 μM)] in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.8 (Tris buffer) were treated as follows before affinity chromatography on plasminogen-substituted Sepharose 4B: Sample 1: 0.5 ml Tris-buffer was added. Sample 2: 0.5 ml Trasylol (5.0 μM) in Tris buffer was added (final concentration 4.5 μM). Sample 3: 0.5 ml Trasylol (5.0 μM) in Tris buffer and 25 μl carboxypeptidase B solution (total 20 U, hippuryl-L-arginine as substrate) were added. Sample 4: 0.5 ml plasmin (1.0 μM) in Tris buffer was added. After incubation for 1 h at 20°C, 0.5 ml Trasylol (5.0 μM) was added to inhibit totally the plasmin activity (5-times molar excess). Sample 5: this sample was first treated exactly as sample 4 and then 25 μl carboxypeptidase B solution (total 20 U) was added and the sample incubated for 20 h at 4°C.

2.3 *Chromatography on plasminogen-substituted Sepharose 4B*

5 ml of samples 1–5 were applied to plasminogen columns (0.28 cm^2 × 2 cm) each containing approx. 5.6 nmol plasminogen. The eluates were collected in volumes of 0.5 ml (fractions 1–10). Elutions were continued using 5 ml Tris buffer (fractions 11–20), 1 ml of 0.2 M 6-aminohexanoic acid in Tris-buffer (fractions 21–22) and finally 2 ml Tris buffer (fractions 23–26).

On a molar basis the columns thus were overloaded with fibrinogen material.

2.4. *Absorbance measurements*

Light absorbance measurements of the eluted fractions were performed at 280 nm using a Beckman 35 spectrophotometer.

2.5. *Rocket immunoelectrophoresis*

Rocket immunoelectrophoresis experiments were performed as described in [6]. 5 μl of the eluted fractions 1,3,5,7,9,11,13,15,17,22 and 25 of the affinity chromatography experiments were applied to rocket immunoelectrophoresis against anti-fibrinogen (1.5 μl/ml agarose gel), and those of the experiment on sample 4 also against anti-FDP E and anti-FDP D (both 1.0 μl agarose gel).

3. RESULTS AND DISCUSSION

A number of affinity chromatography experiments on plasminogen-substituted Sepharose 4B of fibrinogen, plasmin- and/or carboxypeptidase B-cleaved fibrinogen were performed. Figs. 1–3 illustrate the elution patterns obtained. Plasmin catalysed hydrolysis of fibrinogen results primarily in the formation of fibrinogen fragments with C-terminal lysine residues, since plasmin preferentially cleaves Lys-Xaa bonds of fibrinogen [7]. Carboxypeptidase B cleaves off C-terminal lysine and arginine residues [8]. Figs 1–3 show that fibrinogen material treated with carboxypeptidase B is washed off the plasminogen columns with buffer, but no such material is eluted with 6-aminohexanoic acid. After washing, only fibrinogen material not treated with carboxypeptidase B is bound to plasminogen and eluted with 6-aminohexanoic acid. Apparently the C-terminal lysine residues are important for the tight binding of the fibrinogen fragments to plasminogen. Although the results do not exclude a possible importance of C-terminal arginine residues it is very

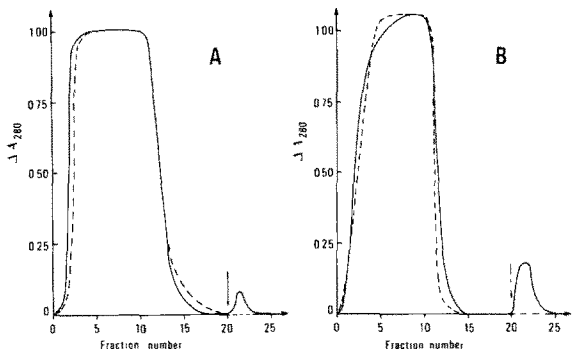

Fig. 1. Elution patterns of affinity chromatography experiments of fibrinogen material on plasminogen columns. (A) Fibrinogen [sample 1 (——)], (sample 2 gave an identical elution pattern). Carboxypeptidase B-cleaved fibrinogen [sample 3 (– – –)]. (B) Fibrinogen fragments (plasmin produced) [sample 4 (——)]. Carboxypeptidase B-cleaved fibrinogen fragments (plasmin produced) [sample 5 (– – –)]. Plasminogen columns: 0.28 cm^2 × 2 cm, 0.56 ml, approx. 5.6 nmol plasminogen each. Samples: 5 ml, 8–9 nmol fibrinogen material. Wash: 5 ml buffer. 0.2 M 6-aminohexanoic acid in buffer: 1 ml (arrows). Buffer: 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.8, 20°C. Collected fractions: 0.5 ml.

unlikely, since plasminogen is known to bind strongly to α-*N*-substituted lysine residues (e.g. lysine Sepharose [9]), and not to α-*N*-substituted arginine residues (e.g. arginine Sepharose [10]).

Native fibrinogen contains no C-terminal lysine residues [7], but the purified fibrinogen used here apparently does (fig.2). It has been reported [11] that partial cleavage of native fibrinogen at the Lys-584–Met-585 bond of the Aα-chain with the release of a small fragment (27 amino acids) occurs already in the circulating fibrinogen. The presence of an impurity with C-terminal lysine residue is thus expected and may explain the results on purified fibrinogen not treated with plasmin (fig.2).

Plasminogen apparently binds fibrinogen fragment E more strongly than it does fibrinogen fragment D (fig.3). The apparent strength of the interactions with plasminogen may reflect the number of C-terminal lysine residues on each fragment. Fibrinogen fragment E carries C-terminal lysine residues on all 3 peptide chains, whereas the various fibrinogen fragments D may carry none, one or two C-terminal lysine residues [7].

The are a great number of reports in the literature on the binding of various peptides and proteins to plasmin(ogen). It is worth noting that several of these are known to have C-terminal lysine residues, particularly peptides obtained by

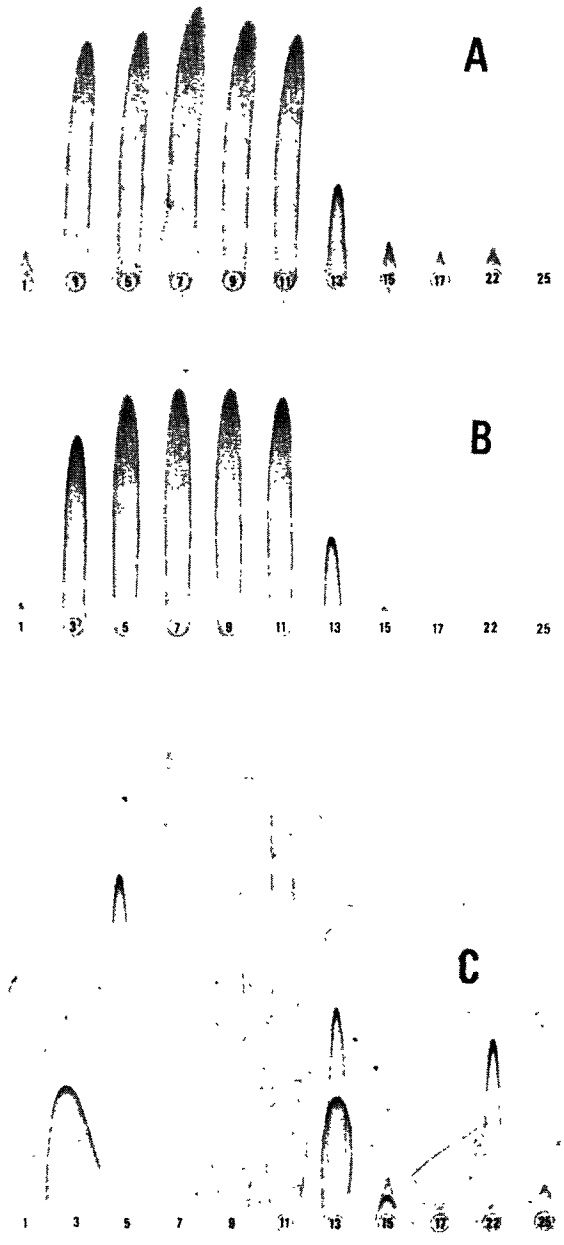

Fig.2. Rocket immunoelectrophoresis against anti-human-fibrinogen of the eluted fractions 1,3,5,7,9,11, 13,15,17,22 and 25 from the plasminogen affinity chromatography experiments. (A) Fibrinogen (sample 1), (B) carboxypeptidase B-cleaved fibrinogen (sample 3), (C) fibrinogen fragments (plasmin produced) (sample 4), (D) carboxypeptidase B-cleaved fibrinogen fragments (plasmin produced) (sample 5). 5 μl of each fraction was applied.

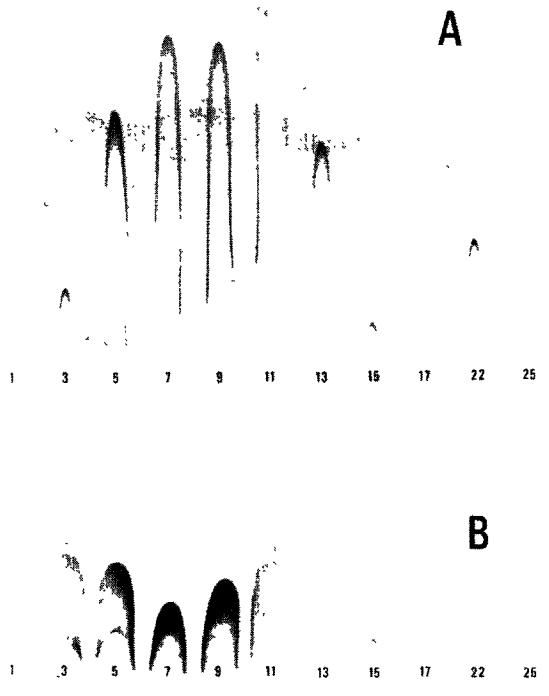

Fig.3. Rocket immunoelectrophoresis of the eluted fractions 1,3,5,7,9,11,13,15,17,22 and 25 from the plasminogen affinity chromatography experiment of fibrinogen fragments (plasmin produced) (sample 4) against (A) anti-human-fibrinogen degradation product E (anti-FDP E), and (B) anti-human-fibrinogen degradation product D (anti-FDP D). 5 μl of each fraction were applied.

cleavage of native proteins with trypsin or plasmin. Several cases of specific interaction between plasmin(ogen) and a particular stretch of peptide in a protein have been claimed from the formation of tight complexes of plasmin(ogen) and the peptide after cleavage with trypsin or plasmin. Such a peptide is perhaps bound to plasmin(ogen) only because of its C-terminal lysine residue, which is not present in the intact protein.

If, as the result presented here indicate, the C-terminal lysine residues of fibrinogen fragments are essential for high-affinity binding of the fragments to plasminogen, then any protein or peptide with C-terminal lysine residue may bind plasminogen and be a modulator of enzyme systems that involve plasminogen. Also, carboxypeptidase N, the plasma C-terminal lysine and arginine hydrolase equivalent to carboxypeptidase B, may affect such systems.

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