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Structure and formation of microplasmin

(plasmin autolysis/disulfide-exchange reactions)

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ABSTRACT **The structure of human microplasmin, prepared from plasmin in alkaline solution, has been studied. Microplasmin consists of two polypeptide chains connected by disulfide bonds. One polypeptide is the B chain of plasmin consisting of 230 amino acids, and the other peptide is the COOH-terminal portion of the A chain of plasmin consisting of 31 amino acid residues. Microplasmin has a molecular weight of 28,635, calculated from its primary sequence. It is slightly more positively charged than plasminogen and is a more hydrophobic molecule. The proposed scheme for the formation of microplasmin involves autolysis at specific peptide bonds and scrambling of especially sensitive disulfide bonds in alkaline solution.**

Plasmin, a trypsin-like serine protease, catalyzes the hydrolytic cleavage of peptide bonds at the COOH sides of arginines and lysines in protein and peptide substrates (1). The enzyme undergoes cannibalistic hydrolysis, plasmin molecules serving as both substrate and enzyme. The autolytic cleavage of plasmin at pH 11.0 leads to the formation of microplasmin (2). Studies of the primary structure of microplasmin and the possible mechanism of its formation are discussed in this report.

MATERIALS AND METHODS

Protein. Microplasmin was prepared from human plasmin variant 2 and purified by affinity chromatography as described (2).

Reduction and *S*-Carboxymethylation. Lyophilized protein (10 mg) was dissolved in 10 ml of 6 M guanidine hydrochloride/0.25 M Tris·HCl/3 mM EDTA/100 μl of 2-mercaptoethanol, pH 8.6, and capped under nitrogen, and the reaction was initiated by the addition of 1.0 ml of a freshly prepared solution of iodoacetic acid (270 mg/ml) and 1.0 M NaOH. The *S*-carboxymethylation was allowed to continue for 30 min in the dark, at which time 100 μl of 2-mercaptoethanol and 12 ml of glacial acetic acid were added. The mixture was applied to a Sephadex G-10 column (1.6 × 90 cm) and eluted with 50% (vol/vol) acetic acid.

Peptide Analysis with HPLC. HPLC was carried out on a μBondapak phenylalkyl column (0.4 × 30 cm) in a Hewlett–Packard model 1090 system. The acetonitrile gradient systems used are described in the legend of each chromatogram. Peptide fractions were pooled and lyophilized. The quantity of peptide in each peak was determined by amino acid analysis.

Amino Acid Analysis. Analyses were carried out after 6 M HCl hydrolysis (24 and 72 hr) with a Beckman amino acid analyzer model 6300.

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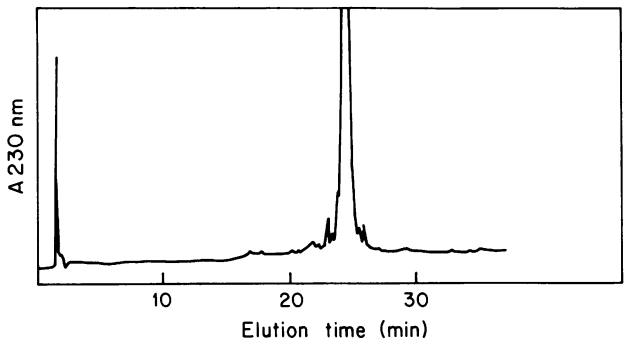

FIG. 1. HPLC chromatography of microplasmin. Microplasmin (100 μg) was denatured in 6 M guanidine hydrochloride and injected onto a μBondapak phenylalkyl column (0.4 × 30 cm) using a linear acetonitrile gradient. Solvent A was 0.05% trifluoroacetic acid in water; solvent B was 0.05% trifluoroacetic acid in acetonitrile; and sample volume was 100 μl. The detector was set for 230 nm. The gradient was 0% solvent B to 60% (vol/vol) solvent B over 1.0 hr at a flow rate of 1 ml/min.

Sequence Analysis. Sequence determinations were carried out by Edman degradation in a Beckman liquid-phase sequencer model 890C that used a 0.1 M Quadrol program.

Reagents and Chemicals. Acetonitrile was obtained from Merck. Guanidine hydrochloride was obtained from Sigma and purified by recrystallization from hot methanol. Trifluoroacetic acid was "sequenal" grade obtained from Pierce. All other chemicals were analytical reagent or "sequenal" grade.

RESULTS AND DISCUSSION

The microplasmin peptides were analyzed on a phenylalkyl HPLC column. One protein peak was detected in the HPLC chromatogram of microplasmin after denaturation with 6 M guanidine hydrochloride (Fig. 1). However, after reduction and *S*-carboxymethylation, the HPLC chromatogram had two polypeptide peaks (Fig. 2). The amino acid compositions of peak I and II are summarized in Table 1. Peak I is identical in composition to that of the polypeptide chain of plasmin from Lys-530 to Arg-560 (Table 1, column 2). Peak II has an amino acid composition identical to that of the intact B chain from Val-561 to Asp-790 (Table 1, column 3). Microplasmin thus has an identical amino acid composition to the plasminogen sequence between Lys-530 and the COOH-terminal Asp-790 (Table 1, column 1). The molar ratio of the peptides in peak I to peak II is 1:1 as calculated from the above results. It is, therefore, proposed that microplasmin consists of two polypeptide chains, peptide I (residues from Lys-530 to Arg-560) (from the A chain) and peptide II (residues from

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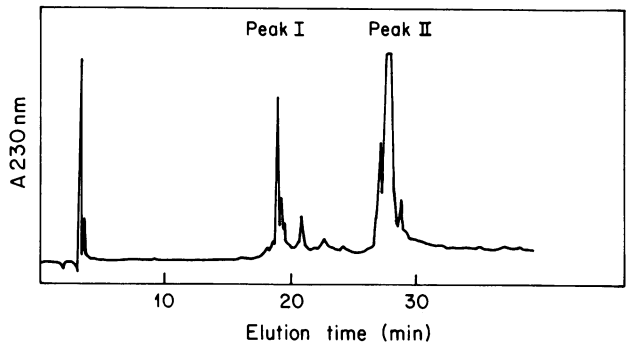

FIG. 2. HPLC chromatography of reduced and carboxymethylated microplasmin. Chromatographic conditions were as in Fig. 1.

Val-561 to Asp-790) (the B chain) connected by disulfide bonds.

Primary structure determination of microplasmin is consistent with the results of the amino acid composition data. The NH_2-terminal sequence analysis of peptide I confirms that peptide I is the peptide fragment of plasminogen between Lys-530 and Arg-560, that is, the 31 residue peptide at the COOH terminus of the A chain of plasmin (Table 2, column 1). The NH_2-terminal sequence of polypeptide II confirms that polypeptide II is the plasmin peptide beginning at Val-561 (Table 2, column 2). Only two peptide sequences were found in microplasmin. We also sequenced a microplasmin preparation that was stored frozen for 3 months prior to analysis. This preparation had two NH_2 termini at residues Leu-531 and Glu-698 in addition to the original ones at Lys-530 and Val-561. These experiments indicate that the cleavage of the peptide bond between Arg-529 and Lys-530 at pH 11.0 is very specific and that hydrolysis of other peptide bonds in microplasmin proceeds at relatively negligible rates.

A possible reaction scheme for microplasmin formation is proposed, as shown in Fig. 3. The peptide bond between Arg-529 and Lys-530 of the plasmin molecule was hydrolyzed by plasmin in step 1 and the disulfide bonds between Cys-511 and Cys-535 and between Cys-461 and Cys-540 undergo rearrangement in step 2.

Two control experiments have been conducted to confirm that plasmin, not hydroxide ion, catalyzed the hydrolysis of the peptide bond between Arg-529 and Lys-530. (*i*) Peptide bond cleavage does not occur in plasminogen upon incubation at pH 11.0. (*ii*) There is neither peptide bond cleavage nor microplasmin formation when 0.1 M benzamidine is added to the plasmin solution at pH 11.0. Therefore, the hydrolysis of the peptide bond between Arg-529 and Lys-530 is catalyzed specifically by plasmin but not by hydroxide ion. Disulfide splitting and exchange in alkaline solution has been observed in studies with bovine serum albumin dimers (4, 5). Cys-511 is in a hydrophilic peptide region and is probably on the protein surface. It is possible that Cys-511 reacts first with hydroxide ion and then the disulfide bond between Cys-511 and Cys-535 can be split. The disulfide bonds between Cys-511 and Cys-535 and between Cys-461 and Cys-540 in plasmin are very close in the sequence and located in the same "kringle" structure. The exchange reactions of these disulfide bonds is possible under these circumstances. The disulfide bond exchange reaction would result in the segregation of microplasmin from the cleaved A chain.

The reaction of plasmin in alkaline solution can serve as a good model for studies of the disulfide exchange reactions and can be a valuable tool for understanding the mechanism of such exchange reactions in proteins. Further study of microplasmin, which contains mainly B chain and no "kringle" structure, and other plasmin fragments should be useful for advancing our knowledge of protein structure and of structure–function relationships in the plasminogen–plasmin system.

Table 1. Amino acid composition of human microplasmin and its peptide fragments after reduction and *S*-carboxymethylation

Amino acid	Residues, no. per mol of microplasmin*	Residues, no. per mol of peak I†	Residues, no. per mol of peak II*
S-Cm-Cys	13.05 (14)‡	3.52 (4)‡	9.87 (10)‡
Asp	17.38 (17)	3.49 (3)	14.70 (14)
Thr§	12.21 (13)	0.40 (0)	12.66 (13)
Ser§	15.21 (16)	1.08 (1)	13.34 (15)
Glu	26.33 (26)	3.09 (3)	23.59 (23)
Pro	20.93 (20)	5.00 (5)	15.90 (15)
Gly	25.58 (25)	2.50 (2)	23.00 (23)
Ala	15.61 (15)	2.00 (2)	13.25 (13)
Val	22.86 (25)	2.00 (2)	21.03 (23)
Met	2.40 (2)	0.00 (0)	1.93 (2)
Ile	8.53 (10)	0.00 (0)	9.12 (10)
Leu	22.23 (22)	0.98 (1)	22.00 (21)
Tyr	6.95 (7)	2.00 (2)	5.22 (5)
Phe	9.37 (9)	1.08 (1)	8.17 (8)
Lys	13.51 (14)	3.52 (4)	10.20 (10)
His	7.00 (7)	0.00 (0)	7.00 (7)
Arg	12.69 (13)	1.12 (1)	12.00 (12)
Trp	ND (6)	ND (0)	ND (6)
Total	(261)	(31)	(230)

The results represent mol of amino acid per mol of peptide after hydrolysis with 6 M HCl/0.1% phenol at 110°C. ND, not determined.
*The composition was normalized to seven residues of histidine. Values are average of 24 samples hydrolyzed for 72 hr unless noted otherwise.
†The composition was normalized to two residues of alanine.
‡The number of residues per mol of peptide was calculated based on the amino acid sequence data of plasmin from residues Lys-530 to Asp-790, from Lys-530 to Arg-560, and from Val-561 to Asp-790 (3).
§Determined from linear extrapolation to zero-hour hydrolysis.

Table 2. NH_2-terminal sequence analysis of microplasmin peptides

Cycle no.	Peak I Amino acid	Peak I Residues, nmol	Peak II Amino acid	Peak II Residues, nmol
1	K	4.5	V	8.0
2	L	4.5	V	7.8
3	Y	3.5	G	3.9
4	D	2.1	G	4.5
5	Y	2.7	CMC	5.0
6	CMC	0.9	V	2.9
7	D	2.4	A	5.3
8	V	1.5	H	1.6
9	P	1.4	P	5.3
10	Q	0.6	H	1.2
11	CMC	0.75	S	0.16
12	A	1.1	W	2.6
13	A	1.8	P	3.0
14	P	0.6	W	1.7
15	S	0.35	Q	3.5
16	F	0.6		

The single-letter amino acid code is used. CMC, carboxymethylated cysteine.

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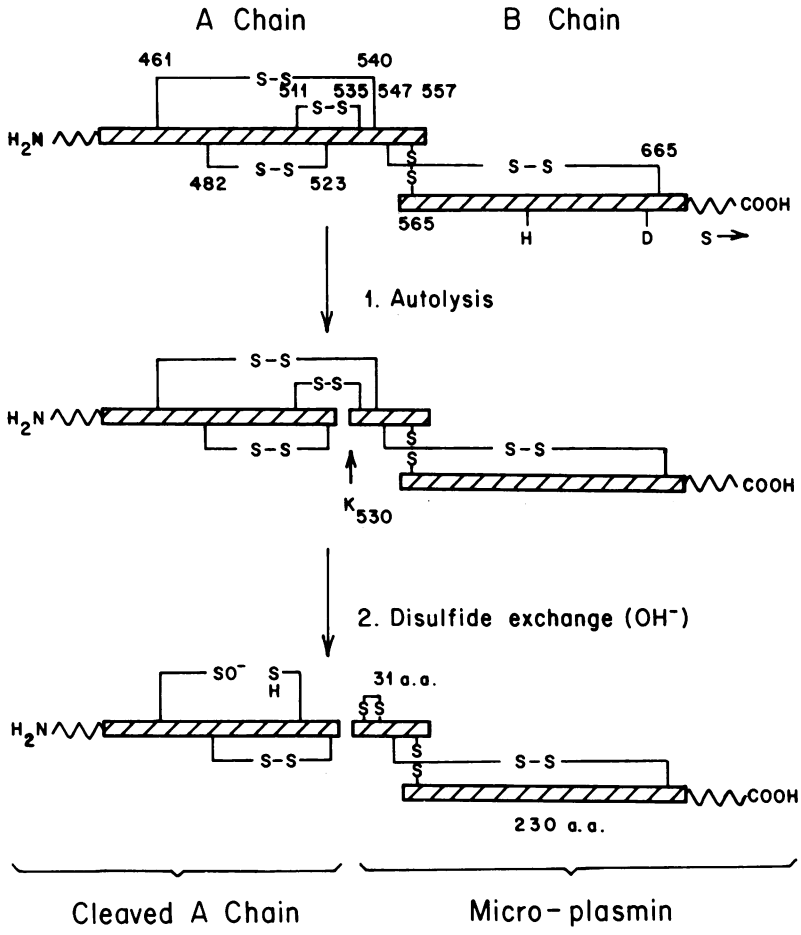

FIG. 3. Reaction scheme for microplasmin formation. a.a., Amino acids.

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