Vol. 98, No. 4, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 27, 1981 Pages 1108-1114

PLASMIN: PHOTOAFFINITY LABELING OF A LYSINE-BINDING SITE WHICH REGULATES CLOT LYSIS

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Received January 19,1981

SUMMARY: Human plasmin has been photoaffinity-labeled by 4-azidobenzoylglycyl-L-lysine. When a solution of plasmin and this peptide is irradiated, the clot-lysis activity is markedly reduced, with only a small loss in activity toward an active-site titrant. Studies with radioactive peptide show that the label is incorporated into the heavy chain of plasmin, with little incorporation into the light chain containing the active site. Plasminogen has also been photoaffinity-labeled by using this reagent. The modified plasminogen can be activated to a plasmin with both active-site titrant and clot-lysis activity. These results establish the presence in plasmin of a lysine-binding site, distinct from that in plasminogen, which regulates clot lysis.

Plasmin is a trypsinlike enzyme whose main physiologic role appears to be the dissolving of fibrin clots. Certain compounds, such as C-terminal lysine peptides, EACA, and trans-4-aminomethylcyclohexane carboxylic acid, affect the activity of plasmin. EACA at 0.3 mM accelerates the hydrolysis of α-N-benzoyl-L-arginine ethyl ester, while a higher concentration (58 mM) inhibits the enzyme (1). These compounds are noncompetitive inhibitors of fibrinolysis by plasmin (2, 3). EACA also blocks the inhibition of plasmin by α_2-plasmin inhibitor (4). These effects have been explained by a model of plasmin in which lysine and its analogs bind both at the active site and at one or more separate allosteric lysine-binding sites, which are involved in binding fibrin (1, 2).

These compounds also affect certain properties of plasminogen, particularly the rate of activation to plasmin. Affinity chromatography studies have shown that the lysine-binding sites in plasminogen are in the part of the plasminogen

The abbreviations used are: NPGB, p-nitrophenyl-p'-guanidinobenzoate; TLCK-plasmin, L-1-chloro-3-tosylamido-7-amino-2-heptanone-inactivated plasmin; EACA, ε-aminocaproic acid; L-BAPA, benzoyl-L-arginine p-nitroanilide.

0006-291X/81/041108-07$01.00/0

chain which, upon activation, becomes the N-terminal portion of the heavy chain of plasmin (4). It has been proposed by several workers, on the basis of binding studies, that there are five lysine-binding sites, including one "strong" binding site, in the amino-terminal "kringle"-containing portion of the heavy chain (5, 6).

This paper reports the photoaffinity labeling of human plasmin by 4-azidobenzoylglycyl-L-lysine at a lysine-binding site which regulates fibrin clot lysis. Human plasminogen has also been photoaffinity-labeled by using this reagent but at a different site.

EXPERIMENTAL PROCEDURES

Human plasminogen was isolated from Cohn Fraction III paste by a modification of the procedure of Liu and Mertz (7). Human Cohn Fraction III pastes were obtained as generous gifts from Dr. Fred Feldman, Armour Pharmaceutical Company. The plasminogen was activated to plasmin with urokinase by the method of Robbins and Summaria (8). Plasmin preparations were 65-70% active by NPGB (9) titration. TLCK-plasmin was prepared by the method of Robbins and Summaria (8) and further purified by chromatography on L-lysine-coupled Sepharose 4B. Plasminogen for photoaffinity labeling was isolated from outdated human plasma by using the procedure of Markus et al. (6) to maintain the plasminogen as native.

Synthesis of Glycyl-ε-tBOC-L-lysine. ε-t-BOC-L-lysine (Chemalog) was coupled with N-benzyloxycarbonylglycine (Peninsula Chemicals) in tetrahydrofuran at -12°C by the procedure of Izumiya et al. (10). The solution was acidified to pH 3.0 with 10% hydrochloric acid and the product extracted with ethyl acetate. Evaporation of the ethyl acetate, left a pale yellow oil. The N-benzyloxycarbonyl group was removed by catalytic hydrogenation at room pressure over 10% palladium on charcoal in methanol:acetic acid:water (50:2:7). After removal of the catalyst by filtration, the solvent was rotary evaporated. The residue was dissolved in water and lyophilized. The resulting white solid gave two spots on analysis by tlc (silica gel/1-butanol:acetic acid:water, 4:1:1). The main spot was yellow to ninhydrin, indicative of N-terminal glycine, while a small contaminant was purple to ninhydrin. This material was used without further purification.

Synthesis of 4-Azidobenzoylglycyl-L-lysine hydrochloride. Glycyl-ε-t-BOC-L-lysine acetate (565 mg, 1.56 mmol) was treated with the N-hydroxysuccinimide ester of 4-azidobenzoic acid (400 mg, 1.54 mmol) (11) in dimethyl sulfoxide containing triethylamine (5.4 mmol). After 23 h at room temperature the reaction mixture was poured into 200 ml of water. The pH was adjusted to 3.0 with 10% hydrochloric acid and the solution extracted three times with 100 ml of ethyl acetate. The combined extracts were evaporated to yield 943 mg of a clear oil. The ε-t-BOC group was removed with 4.2 N HCl in ethyl acetate at room temperature. The product was purified by chromatography at 2°C on a Sephadex G-10 column (2.0 x 85 cm) with 0.17 M acetic acid as solvent. Appropriate fractions were pooled and lyophilized to a white solid. The material gave a single spot, ninhydrin-positive (purple) and ultraviolet-positive, on analysis by tlc (silica gel/1-butanol:acetic acid:water, 4:1:1). The yield was 134 mg, 0.33 mmol, 22%. Elemental analysis (Galbraith Laboratories, Inc.) was correct for 4-azidobenzoyl-glycyl-L-lysine monohydrochloride monohydrate. Anal. Calcd for $C_{15}H_{23}N_6O_5Cl$: C, 44.72; H, 5.75; N, 20.86. Found: C, 44.67; H, 5.72; N, 20.60. Amino acid

analysis of a hydrolyzed sample gave glycine and lysine in a 1-to-1 ratio. UV max (0.3 M PO_4, pH 7.6) 270 nm (ε = 22,879).

The 4-azido[carbonyl-^{14}C]benzoylglycyl-L-lysine monohydrochloride monohydrate was synthesized in an analogous manner by using 4-amino[carboxyl-^{14}C]benzoic acid (ICN) as precursor for the 4-azidobenzoic acid. The radioactive peptide had 393,418 cpm/μmol.

Photolysis Experiments. Prior to the photolysis experiments the plasmin solution was passed over a Sephadex G-25 column (2.0 x 40 cm) equilibrated in 0.3 M potassium phosphate buffer, pH 7.6, at 2°C. After addition of azidopeptide and 10% glycerol the sample (6 ml, ca 2 x 10^{-5} M active sites) was assayed for clot-lysis activity by the method of Carlin and Saldeen (12). Active site concentrations were determined by using NPGB (9). Photolysis experiments were carried out at 6°C in a Raynet Photochemical Reactor equipped with 300-nm lamps. A polystyrene test tube and Pyrex cooling vessel were used to eliminate light of wavelength <295 nm (13). After 30-min irradiation the solution was again assayed for NPGB and clot-lysis activity. TLCK was then added to inhibit the enzyme and prevent autolysis in the subsequent work-up.

The sample was freed of noncovalently bound labeling reagent and photoproducts by passage over a Sephadex G-25 column (2.0 x 40 cm). Pertinent fractions were pooled and applied to a column of L-lysine-coupled Sepharose 4B (2.0 x 30 cm). Elution was carried out at 2°C at a flow rate of either 12.7 ml cm^{-2} h^{-1} or 22.9 ml cm^{-2} h^{-1} with a gradient from 0.1 M potassium phosphate, pH 7.6, to 0.1 M potassium phosphate--12 mM EACA, pH 7.6. Aliquots of fractions were counted in Aquasol in a Beckman LS-250 liquid-scintillation system. The concentration of EACA in a given fraction was determined by precipitating the protein of a suitable aliquot with 16% trichloroacetic acid and reacting the supernatant with ninhydrin, according to the procedure of Moore (14). Protein concentrations were determined by the method of Beardon (15). The plasmin chains were reduced, alkylated, and separated by the dialysis method of Groskopf et al (16).

RESULTS AND DISCUSSION

Properties of Labeling Reagent. 4-Azidobenzoylglycyl-L-lysine is a competitive inhibitor of the hydrolysis of L-BAPA by plasmin with a K_i = 6.3 x 10^{-3} M. In the absence of ultraviolet light the azidopeptide inhibits clot lysis with an I_{50} = 8.0 x 10^{-5} M. When irradiated the azidopeptide decomposes with a half-life of 7 min, as measured by loss of absorbance at 270 nm. Ultraviolet light converts the 4-azidobenzoyl group to a reactive nitrene capable of insertion into any available bond.

Effect of Photoaffinity Labeling on Plasmin Activity. The effects of photoaffinity labeling on the number of functional active sites and on the clot-lysis activity of plasmin are presented in Table 1. The slight loss of NPGB activity may be due to minor labeling of the active site of plasmin, since plasmin in 10% glycerol without azidopeptide was stable under the photolysis conditions. With-

Table 1. EFFECT OF PHOTOAFFINITY LABELING ON PLASMIN ACTIVITY

Azidopeptide/ Plasmin Ratio[a]	% Initial Activity NPGB	% Initial Activity Clot Lysis	Mol Label/ Mol Protein
0	100	90	----
40	96	33	0.55
40	86	38	0.75
20	84	37	0.62
12	88	23	0.73
11	87	23	0.65
44 + 0.1 M EACA	99	40	0.60
41 + 0.05 M BzGlyLys	90	79	0.29

[a] Plasmin ca 2 x 10^{-5} M.

out glycerol the photolysis conditions cause partial loss of clot-lysis activity (ca 25%) but no loss of NPGB activity.

The clot-lysis activity of plasmin is markedly reduced by the photoaffinity labeling. This loss of activity was somewhat variable between experiments and was never total, even at the highest azidopeptide/plasmin ratios used. The inconsistent results of labeling are probably due in large part to the highly reactive and unstable nature of the nitrene intermediate. However, the approximate correlation between amount of label incorporated and residual clot-lysis activity suggests that one lysine-binding site is being labeled. This suggestion is supported by competition experiments with benzoylglycyl-L-lysine, which affords a high degree of protection against inactivation by the photoaffinity-labeling reagent. EACA, in contrast, is a poor protecting agent. Each inhibitor gives a sum of residual clot-lysis activity and molar label/enzyme ratio of near unity.

Effect of Photoaffinity Labeling on Binding of Plasmin to L-lysine-Coupled Sepharose 4B. Plasmin chromatographs as two peaks on L-lysine-coupled Sepharose 4B, when an EACA gradient is used to elute the protein (17, 18). All of the photoaffinity-labeled plasmins eluted as two components on the affinity column (Fig. 1) but with a greatly reduced affinity, as indicated by the concentrations of EACA required to elute them (Table 2). In most cases the labeled forms are

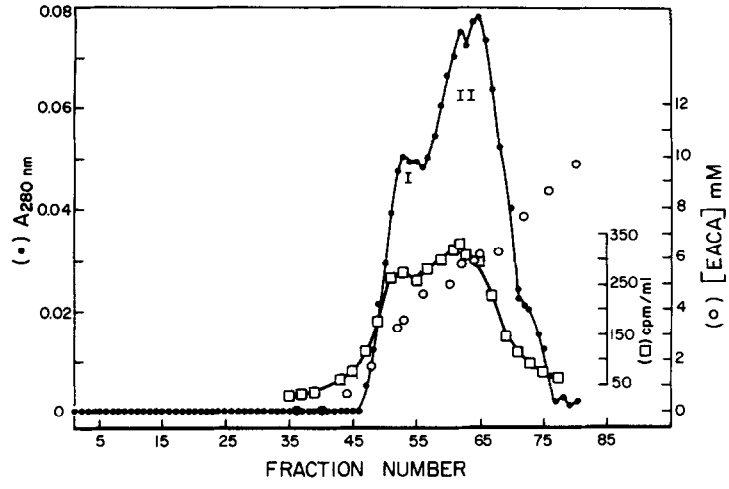

Fig. 1. Chromatography of photoaffinity-labeled plasmin on lysine-coupled Sepharose (azidopeptide/plasmin = 40).

not completely resolved; the first component (I) appeared as a shoulder on the peak for the second form (II). The two peaks apparently represent the original affinity forms of plasmin, modified at a lysine-binding site. However, peak II may contain a mixture of labeled and unlabeled plasmin, since no peaks for

Table 2. EFFECT OF PHOTOAFFINITY LABELING ON BINDING OF PLASMIN AND PLASMINOGEN TO LYSINE-COUPLED SEPHAROSE[a]

Protein	Azidopeptide/ Plasmin Ratio	EACA (mM) I	EACA (mM) II	CPM/A_{280} I	CPM/A_{280} II
Plasmin		6.2	10.3		
TLCK-plasmin		6.8	11.3		
[^{14}C]plasmin	40	3.6	5.8	5,244	4,297
	40	2.9	5.4	3,830	3,221
	20	3.4	6.1	3,147	2,529
	12	3.3	7.3	3,007	2,675
	11	4.2	6.8	3,285	3,126
	44[b]	3.7	6.0	2,327	2,233
	41[c]	7.0	12.0	1,813	1,074
Plasminogen		5.7	10.0		
[^{14}C]plasminogen[d]	40	4.1[e]			
	40	4.8[e]			

[a] I, II = first and second elution peaks.
[b] Plus 0.1 M EACA, a competitive inhibitor.
[c] Plus 0.05 M BzGly-L-Lys, a competitive inhibitor.
[d] Contained 1.2 mol label/mol plasminogen.
[e] Only one form was observed.

unlabeled plasmin were observed to account for the residual clot-lysis activity. Peak II consistently averaged a lower cpm/A_{280} than Peak I.

Location of Labeled Sites within Plasmin Molecule. Photoaffinity-labeled plasmin was reduced and carboxymethylated, and the chains were separated (16). The heavy chain contained 18,238 cpm/mg; the light chain, containing the active site, had 2,014 cpm/mg. Heavy chains isolated by this procedure always contain trace amounts of contaminating light chain, as determined by gel electrophoresis; but since the incorporation into the light chain is low, this contamination does not alter the conclusion that the site being labeled is in the heavy chain.

Effect of Photoaffinity Labeling on Plasminogen. Native plasminogen displays two peaks when chromatographed on L-lysine-substituted Sepharose (Table 2). When plasminogen is photoaffinity-labeled with 4-azidobenzoylglycyl-L-lysine, the resulting plasminogen displays only one form, with a reduced affinity for L-lysine-coupled Sepharose. Photolabeled plasminogen is readily activated to plasmin by urokinase. Although activation conditions were not optimized, labeled plasminogen yielded 67-86% of the plasmin activity of a comparable sample of unmodified plasminogen. The active plasmin had 67-80% of the clot-lysis activity of unmodified plasmin.

The results presented here confirm, both by direct covalent modification of plasmin and by correlation of loss of activity with modification, the role of the lysine-binding sites in clot lysis and their presence in the heavy chain of plasmin. Experiments with competitive inhibitors indicated that most of the specificity observed in photoaffinity labeling is due to specific binding. Presumably the one site being labeled in native plasminogen is the one strong lysine-binding site reported by Markus et al. (6) and located in the first kringle structure by Lerch et al. (19). The fact that photolabeled plasminogen can be activated to a plasmin with clot-lysis activity suggests that the site being modified in plasminogen is different from that labeled in plasmin. The site photolabeled in plasminogen apparently is not required for the binding of fibrin as a sub-

strate by plasmin. Further studies are in progress to isolate the peptides containing these two labeled sites and to locate the modified residue(s) within the plasminogen molecule.

Acknowledgments. I thank Ms. Mary E. Carello for her excellent technical assistance. I am grateful to Dr. Thomas H. Plummer, Jr., for helpful discussions and advice about this work.

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