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Mapping of the Human Plasmin Domain Recognized by the Unique Plasmin Receptor of Group A Streptococci

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A high-affinity surface receptor for human plasmin has been reported on certain group A streptococci. To map the region of the plasmin molecule that binds to the bacterial receptor, isolated domains of plasmin were tested for their ability to inhibit the binding of intact radiolabeled plasmin to receptor-positive bacteria. Complete inhibition of binding of labeled plasmin to bacteria by isolated heavy chains was achieved, but this inhibition was not as efficient on a molar basis when compared with that of unlabeled plasmin. By contrast, a conformationally altered form of native plasminogen was found to bind to bacteria and was as efficient a competitive inhibitor as intact plasmin was. The results of this study indicate that the selective binding of human plasmin to a group A streptococcus is dependent on structures present in the conformationally altered form of native plasminogen or plasmin that are not found on the native zymogen, the plasminogen with NH_2-terminal glutamic acid.

Recently, we have demonstrated that certain pathogenic group A streptococci grown in either Todd-Hewitt broth or chemically defined media express a receptor that binds to human plasmin, while they demonstrate no significant reactivity with the native zymogen form of the protein, the native human plasminogen with NH_2-terminal glutamic acid (Glu-plasminogen), or with other serine-class proteases (14). Bacterium-bound plasmin retains its enzymatic activity and can no longer be regulated by its physiological inhibitor, α_2-antiplasmin (14). Optimal binding of plasmin to its bacterial receptor has been shown to occur under physiological conditions of ionic strength and pH (3). This interaction of plasmin with a group A streptococcus has a high affinity and an estimated dissociation constant of approximately 1.0×10^{-10} M (3). Plasmin binding is inhibited reversibly by lysine or ε-aminocaproic acid (EACA) (3). These data suggest that the lysine-binding kringle structures (homologous triple-loop structures) of the plasmin molecule might be involved in the association of plasmin with the bacterial receptor. The structure of human plasmin(ogen) is well characterized (Fig. 1), and methods for preparing defined fragments have been reported (16, 21, 23, 31). The purpose of this investigation was to prepare a variety of fragments of plasmin and plasminogen in order to localize the region of the plasmin molecule which interacts with the bacterial plasmin receptor. In this study we report that the binding of plasmin to a group A streptococcus is dependent on the conformation of the plasmin molecule and involves interactions that are distinct from those that occur between other known plasmin(ogen)-binding molecules like streptokinase, fibrin, fibrinogen, thrombospondin, or α_2-antiplasmin.

MATERIALS AND METHODS

Enzymes, inhibitors, and other reagents. Urokinase and porcine elastase (type IV) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Aprotinin was obtained as Trasylol from Mobay Pharmaceuticals (New York, N.Y.). Phe-Pro-Arg-chloromethylketone (PPACK) was obtained from Calbiochem-Behring (San Diego, Calif.). The proteolytically modified form of human Glu-plasminogen with an NH_2-terminal lysine (Lys-plasminogen) was obtained from American Diagnostica Inc. (Greenwich, Conn.). H-D-Val-Leu-Lys-*para*-nitroanilide (S-2251) was obtained from Helena Laboratories (Beaumont, Tex.).

Human plasminogen. Native human plasminogen (Glu-plasminogen) was prepared from human plasma by chromatography on lysine-Sepharose and molecular sieving chromatography on Superose 6 (fast protein liquid chromatography [FPLC]; Pharmacia Fine Chemicals, Piscataway, N.J.). The purified protein appeared as a single band on a silver stain of a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Plasminogen was quantified by measuring the absorbance with an $A_{280}^{1\%,\ 1\ cm}$ value of 17.0 (18). The protein was also quantified antigenically by rocket electrophoresis as described by Laurell (10). The purity of the isolated human plasminogen was confirmed by activation of a known quantity of plasminogen with streptokinase and by measuring the amidolytic activity. The observed and theoretically predicted enzymatic activities were equivalent, within experimental error. Human Lys-plasminogen, a modified form of Glu-plasminogen in which 76 of the NH_2-terminal amino acid residues were removed (Glu-1 to Lys-76), was obtained from American Diagnostica Inc. The homogeneity of this Lys-plasminogen preparation was analyzed by using both a urea gel electrophoresis procedure and an acetic acid-urea gel electrophoresis procedure. This Lys-plasminogen preparation demonstrated the appropriate migratory property (a shift to a lower M_r, approximately 85,000) in comparison with that of the native Glu-plasminogen (M_r, approximately 92,000).

Iodination of proteins. Glu- and Lys-plasminogens were iodinated by the chloramine T method by using Iodobeads (Pierce Chemical Co., Rockford, Ill.) as described by Markwell (17). The labeled proteins were separated from free iodine by passage over a G-25 column (PD-10; Pharmacia) and collected in 0.15 M Veronal (Winthrop Laboratories, Div. Sterling Drug Co., New York, N.Y.)-buffered saline (pH 7.4) containing 0.001 M Mg^{2+}, 0.00015 M Ca^{2+}, and 0.1% gelatin (VBS-gel). All labeled proteins were analyzed for homogeneity by gel electrophoresis and autoradiogra-

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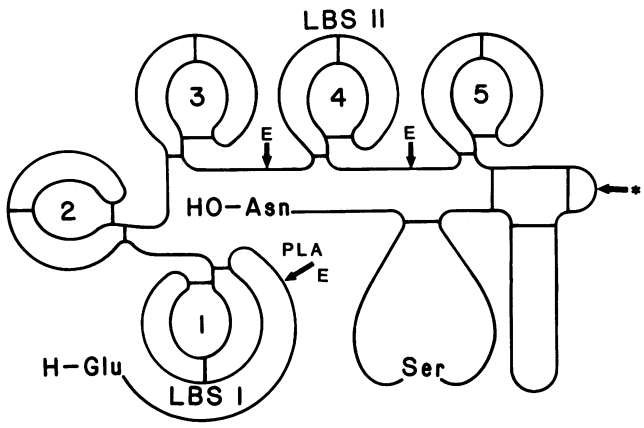

FIG. 1. Schematic representation of the plasminogen molecule. This figure was adapted from Collen (7). The asterisk-labeled arrow indicates the bond that was cleaved during plasminogen activation, and the E- and PLA-labeled arrows indicate elastase and plasmin cleavage sites, respectively.

phy. After labeling, each tracer migrated as a single band in the corresponding position of the unlabeled fragments (for example, see Fig. 4). The labeled proteins were stored in fractions containing 0.02% sodium azide at −20°C. The concentration of ^{125}I-labeled plasminogen was determined antigenically by using a sandwich enzyme-linked immunosorbent assay technique with goat anti-human plasminogen immunoglobulin G fraction from Atlantic Antibodies (Scarborough, Maine). This assay reliably measured plasminogen in the nanogram range.

Generation of plasmin. Lys-plasmin was generated from radiolabeled or unlabeled Glu- or Lys-plasminogen by incubation with urokinase (20 U/ml) in VBS-gel (unless stated otherwise) that contained 0.04 M lysine. The conversion of the single-chain zymogen molecule to the two-chain plasmin enzyme was monitored by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, as described previously (14). Conversion of the zymogen to the active enzyme was maximal after 30 min of incubation at 37°C. Glu-plasmin was generated by a similar procedure, with the exception that a 10-fold molar excess concentration of aprotinin relative to the Glu-plasminogen concentration was added prior to the addition of urokinase (22). Mini-plasmin was generated from mini-plasminogen by using the same activation procedure described above to generate Lys-plasmin. PPACK-reacted radiolabeled or unlabeled plasmin was obtained by mixing a fivefold molar excess of the inhibitor with plasmin and incubating the solution at 37°C for 30 min.

Bacteria. Group A, beta-hemolytic *Streptococcus* strain 64/14 was grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) overnight at 37°C as stationary cultures (35). The bacteria were harvested by centrifugation and suspended in phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 and 0.02% sodium azide. The bacteria were killed by heating them at 80°C for 15 min. Previous studies have demonstrated that this heat treatment does not alter the ability of group A streptococci to bind plasmin (3, 14). The suspension was centrifuged and the pellet was washed twice with VBS-gel containing 0.02% sodium azide. Fractions were stored at −20°C. Stocks of 10% (wt/vol; wet weight) suspensions were prepared in VBS-gel containing 0.02% sodium azide. The concentration of a bacterial suspension was determined by counting the bacterial chains in a hemacytometer (Neubauer; Fisher Scientific, Orlando, Fla.).

PAGE. PAGE was carried out as described by Weber and Osborn (29) with the addition of 6.0 M urea to the polyacrylamide gel. The polyacrylamide gels consisted of a 4% stacking gel layered onto a 10 or 12% polyacrylamide gel containing 6.0 M urea, 0.1% SDS, and 0.05 M sodium phosphate (pH 7.1). Slab gels were used in a Protean II system (Bio-Rad Laboratories, Richmond, Calif.). Protein samples were prepared by mixing an equal volume of sample buffer containing 0.1 M sodium phosphate (pH 7.1), 8.0 M urea, and 4.0% SDS with the protein solution and heating it at 80°C for 2 min. Sample buffer containing 0.72 M β-mercaptoethanol was used to prepare protein samples in the reduced state.

Preparation of elastase digestion fragments of plasminogen. Elastase digestion of human plasminogen yielded three defined fragments of the plasminogen molecule (Fig. 1). These were (i) the lysine-binding domain I (LBS-I) with an M_r of approximately 38,000 containing kringle (homologous triple-loop structures) domains 1 through 3, (ii) lysine-binding domain II (LBS-II) with an M_r of approximately 10,000 to 12,000 consisting of kringle domain 4, and (iii) the non-lysine-binding domain known as mini-plasminogen with an M_r of approximately 36,000 containing the remainder of the heavy chain (kringle domain 5) and an intact light chain. Elastase digestion was performed by using established conditions (21). Purified Glu-plasminogen (3.0 mg/ml) in 0.05 M Tris hydrochloride–0.1 M NaCl (pH 8.0) was digested with a 40:1 molar ratio of Glu-plasminogen to porcine elastase in the presence of 250 Kallikrein inhibitory units (KIU) of aprotinin per ml for 6.5 h at room temperature with gentle stirring in a total volume of 20 ml. At that time a fraction containing 50 μg of protein was removed for analysis by SDS-PAGE and silver staining, to determine the extent of plasminogen digestion. The remainder of the reaction mixture was flash frozen and stored at −70°C. The fragments were subsequently purified by a combination of affinity chromatography on lysine-Sepharose and gel filtration on Superose 6 (FPLC; Pharmacia). The concentrations of the purified proteins (Fig. 2A) were determined spectrophotometrically by using previously reported $A_{280}^{1\%,\ 1\ cm}$ values of 17.0 for both Glu- and Lys-plasminogens (18), 14.0 for mini-plasminogen (9, 18), 22.5 for LBS-I (18), 25.0 for LBS-II (18), and 16.0 for the plasmin heavy and light chains (23). All proteins were divided into fractions and stored at −70°C.

Preparation of plasmin heavy and light chains. Plasmin heavy and light chains were prepared essentially as described by Summaria and Robbins (23). A total of 20 mg of Lys-plasmin, which was enzymatically inhibited with a fivefold molar excess of aprotinin in 5 ml of 0.05 M Tris hydrochloride–0.1 M NaCl (pH 8.0), was reduced by treatment with 0.1 M β-mercaptoethanol for 20 min at 20°C. The reduced solution was then cooled in an ice slurry and carboxymethylated with 0.1 M sodium iodoacetate on ice for 10 min. The plasmin heavy and light chains were then separated and purified by a combination of affinity chromatography on lysine-Sepharose, concentration by ammonium sulfate precipitation (4.0 g/10 ml), and suspension in 0.05 M Tris hydrochloride–0.1 M NaCl (pH 8.0) and were subjected to gel filtration on Superose 6 (FPLC; Pharmacia). The isolated plasmin and plasminogen fragments were analyzed for purity on a reduced SDS–6 M urea-polyacrylamide gel. The various fragments demonstrated appropriate molecular sizes and were homogeneous (Fig. 2B). Concentrations were determined as described above. All proteins were divided into fractions and stored at −70°C.

Direct binding assay of radiolabeled proteins. The ability of radiolabeled plasminogen fragments to bind to group A *Streptococcus* strain 64/14 was measured as described previously (14). A fixed number of bacteria were incubated with labeled proteins (approximately 30,000 cpm per tube) in a total volume of 400 μl of VBS-gel for 30 min at 37°C. The bacteria were pelleted by centrifugation at 1,000 × *g* for 10 min, and the pellets were washed twice with 2.0 ml of VBS-gel. The radioactivity associated with the bacteria was determined in a gamma counter (5500 Auto; Beckman Instruments, Inc., Fullerton, Calif.). All estimates were performed in duplicate.

Inhibition of plasmin binding to bacteria by purified plasmin(ogen) fragments. The ability of different concentrations of one or more of the isolated plasmin(ogen) fragments to inhibit binding of PPACK-reacted, ^{125}I-labeled Lys-plasmin to group A *Streptococcus* strain 64/14 was tested by a modification of the direct binding assay described above. Different concentrations of plasmin(ogen) or plasmin(ogen) fragments were mixed with a fixed dilution of a 10% (wt/vol) suspension of *Streptococcus* strain 64/14 and PPACK-reacted, ^{125}I-labeled Lys-plasmin (approximately 30,000 cpm per tube), followed by incubation for 30 min at 37°C. We have previously established that under these conditions equilibrium is established and that significant ligand is not lost when the bacteria are washed free of unbound ligand (3). Bacterium-associated radioactivity was determined after unbound label was washed away as described above. The inhibition of binding of labeled plasmin was calculated by comparing the number of counts bound in the absence of competitor with the number of counts bound when the competitor was present. All samples were corrected for background binding of counts, i.e., counts bound in the tubes from which bacteria were omitted or in tubes in which a 100-fold molar excess of unlabeled ligand was added. In no case was the background level of radioactivity greater than 5% of the counts offered. Furthermore, background levels in the presence of excess unlabeled competitor or in the absence of bacteria were not significantly different.

Elution and analysis of ^{125}I-labeled Lys-plasmin(ogen) from bacteria. ^{125}I-labeled Lys-plasminogen (approximately 100,000 cpm) was added to a 100-μl fraction of a 10% (wt/vol) solution of strain 64/14 in a total volume of 400 μl of VBS-gel and was allowed to incubate at 37°C for 30 min. The bacteria were then pelleted by centrifugation (3,000 × *g* for 10 min) and washed three times with 2.0 ml of VBS-gel. The bacterial pellets were suspended in 300 μl of VBS-gel containing 0.5% SDS, VBS-gel containing 0.1 M EACA, or VBS-gel containing 0.5% SDS and 2% 2-mercaptoethanol, to elute the ^{125}I-labeled Lys-plasminogen from the bacteria. Following a 10-min incubation at 37°C, the bacteria were removed by centrifugation and the supernatant was recovered. The eluted material was analyzed by electrophoresis on an SDS–6 M urea–10% polyacrylamide gel under reducing conditions. The gel was dried, and the migration of labeled protein was determined by autoradiography. Similar studies were also carried out in which the bacterium-bound ^{125}I-labeled Lys-plasminogen was treated with a 20-U/ml concentration of urokinase for 20 min at 37°C in a total volume of 300 μl of VBS-gel before the bound proteins were eluted. Following this plasminogen activation reaction, the bacteria were centrifuged and washed twice with 2.0 ml of VBS-gel. The residual bound ^{125}I-labeled Lys-plasmin(ogen) was eluted and analyzed as described above.

Measurement of functional activity of plasmin(ogen) in bacterium-free supernatants. The following assay was used to measure the binding of the various plasmin(ogen) species as an alternative method to the use of radiolabeled tracers. In these studies, 2.0 μg of Glu-plasminogen, Lys-plasminogen, or Lys-plasmin was incubated with 100 μl of a heat-killed 10% (wt/vol) suspension of group A *Streptococcus* strain 64/14 for 30 min at 37°C in a total reaction volume of 400 μl of VBS-gel. Following incubation, the bacteria were removed by centrifugation at 12,000 × *g* for 4 min in a microfuge (Eppendorf) and bacterium-free supernatants were obtained. Control tubes for each plasmin(ogen) species containing no bacteria were treated identically, and all samples were run in duplicate. The bacterium-free supernatants were recovered, and enzymatic activity was measured as follows. The bacterium-free supernatants or the corresponding control samples were added to plastic cuvettes containing 10^6 IU of streptokinase in a total volume of 0.9 ml of enzyme assay buffer (0.05 M Tris hydrochloride, 0.05 M NaCl, 0.1% polyethylene glycol 8000 [pH 7.4]). For the Glu- and Lys-plasminogen preparations, the reaction mixture was incubated at 37°C for 10 min to allow plasminogen-streptokinase complexes to form. For Lys-plasmin, a similar incubation with streptokinase was performed to generate plasmin-streptokinase complexes. Following incubation, S-2251 was added to yield a final concentration of 300 μM. Tubes were allowed to incubate for precisely 5 min and then quenched with 100 μl of glacial acetic acid. The amount of substrate hydrolysis, which was directly proportional to the amount of active enzyme present, was then recorded by measuring the A_{405} of the reaction mixture.

The enzymatic activity of the bacterium-free supernatant was determined by comparing it with the enzymatic activities of known standards. The percentage of residual enzymatic plasmin(ogen) activity in the bacterium-free supernatant was calculated by determining the fraction of total enzymatic activity in a control sample that remained in the supernatant following incubation with bacteria. Control tubes containing bacteria and substrate and substrate in buffer were included. All assays were performed in duplicate.

Measurement of functional activity of plasmin associated with bacteria. The plasmin activity associated with bacterial pellets was examined by using the chromogenic substrate as described above. Following binding and centrifugation, the pellets were washed three times with 1.0 ml of VBS-gel and suspended in 400 μl of the enzyme assay buffer. S-2251 was added to yield a final concentration of 300 μM. The suspended bacterial pellets were then incubated at 37°C for 20 min and quenched with 50 μl of glacial acetic acid. The bacteria were removed by centrifugation (12,000 × *g* for 4 min), and the optical density of the bacterium-free supernatant was measured at 405 nm. Control tubes containing bacteria and substrate and substrate in buffer were included. All assays were performed in duplicate.

RESULTS

The experiments described in this report were designed to map the domains on the human plasmin molecule involved in the high-affinity interaction with group A *Streptococcus* strain 64/14. For these studies, a variety of defined plasminogen fragments, as well as the heavy and light chains of plasmin, were prepared as described above. The plasminogen fragments obtained were characterized on urea gels (Fig. 2). The homogeneous plasminogen fragments were used to compete with intact PPACK-reacted, ^{125}I-labeled Lys-plasmin for receptor sites on group A *Streptococcus* strain 64/14

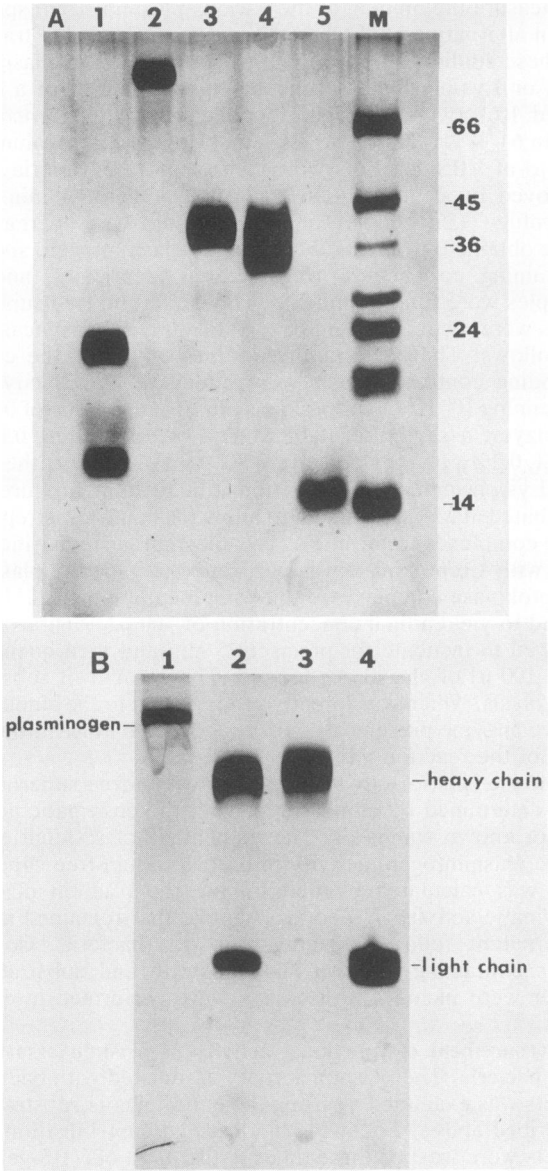

FIG. 2. SDS-PAGE-urea analysis of isolated plasmin(ogen) fragments. (A) Elastase digestion fragments of plasmin(ogen). Glu-plasminogen was digested with elastase, and the fragments were purified as described in the text. Lanes: 1, mini-plasmin (4.0 μg); 2, Glu-plasminogen (4.0 μg); 3, mini-plasminogen (4.0 μg); 4, LBS-I (4.0 μg); 5, LBS-II (4.0 μg); M, molecular weight standards (in thousands). (B) Plasmin heavy- and light-chain preparations. Lys-plasmin was reduced and carboxymethylated as described in the text. Lanes: 1, Glu-plasminogen (5.0 μg); 2, Lys-plasmin (5.0 μg); 3, heavy chain (5.0 μg); 4, light chain (5.0 μg). Proteins were electrophoresed under reducing conditions on an SDS–10% polyacrylamide–6 M urea gel.

(Table 1). We have previously demonstrated that treating plasmin with PPACK, *p*-nitrophenyl-*p*-guanidinobenzoate, or aprotinin does not affect the binding reactivity of plasmin to streptococci (14). Enzymatic inhibition of the ^{125}I-labeled Lys-plasmin and urokinase in the labeled tracer preparation was necessary to prevent the proteolytic conversion of Glu-plasminogen to Lys-plasminogen or Lys-plasmin (16) by the labeled tracer mixture. The quantity of radioactivity bound in the presence or absence of unlabeled competitor was compared, and the degree of inhibition was calculated (Fig. 3). The results summarized in Table 1 indicate that unlabeled plasmin inhibits the binding of labeled plasmin efficiently, with 50% inhibition being observed in the presence of 1.2×10^{-2} μM Lys-plasmin. Significant inhibition of radiolabeled plasmin binding was also observed when purified heavy chain was used as the competitor. The addition of any of the other plasminogen fragments, including isolated lysine-binding domains of the heavy chain (LBS-I or LBS-II), demonstrated no significant inhibitory effect (Fig. 3). Similarly, mini-plasminogen, mini-plasmin, and isolated light chains demonstrated no significant inhibition of binding of radiolabeled Lys-plasmin over the concentration range tested (1 to 10^{-4} μM) (Fig. 3). Identical results were obtained in the inhibition assays involving mini-plasmin, mini-plasminogen, LBS-I, LBS-II, the Lys-plasmin heavy chain, and the plasmin light chain in the absence of protease inhibitors in the reaction mixture (data not shown).

When equimolar quantities of the elastase-digested fragments of plasminogen or plasmin were combined, there was no restoration of any inhibitory potential. Furthermore, a combination of the isolated light chain and the heavy chain demonstrated no synergistic effect in inhibitory capacity compared with the effect of the sum of the isolated fragments alone (data not shown). The inhibition curves for isolated heavy-chain and intact plasmin (Fig. 3) demonstrated that both preparations inhibited binding of labeled Lys-plasmin by 100%. However, these curves differed in shape, indicating differences in the efficiency of inhibition. The isolated heavy chain was found to be less efficient an inhibitor than the intact plasmin molecule was. These findings suggest that there is some component involved in the interaction of plasmin with the bacteria that either is not present on the heavy chain or is altered during the isolation procedure.

Two possibilities to account for these observations were considered. The first was that there are some sites on the heavy chain of the plasmin molecule that are modified when the molecule is purified, thereby changing its efficiency of interaction with the bacterial receptor. The second was that the plasmin light chain, when associated with the heavy chain, confers a different tertiary structure to the molecule than exists on either (or both) of the isolated chains. Such a change in conformation of the molecule might affect its interaction with the bacteria. It has been established previously that a conformational change occurs when Glu-plasminogen is activated to Lys-plasmin or when Glu-plasminogen is converted to Lys-plasminogen (16, 22, 24). Lys-plasminogen is a zymogen form of plasminogen that lacks the 76-amino-acid NH_2 terminus of the native protein. This modification results from the proteolytic activity of plasmin on Glu-plasminogen, which removes the 76-amino-acid NH_2 terminus, resulting in a new NH_2 terminus lysine (for a review, see reference 22). This modification occurs without the generation of protease activity. The conversion of Glu-plasminogen to Lys-plasmin or Lys-plasminogen not only results in a marked conformational change of the protein but also causes an increase in the binding affinity of these molecules to fibrin (24). It also lowers the dissociation constant between these molecules and α_2-antiplasmin (22, 33).

To examine the possible importance of the conformation of the plasmin(ogen) molecule for binding to bacteria, the ability of the conformationally altered form of plasminogen (Lys-plasminogen) to bind to group A *Streptococcus* strain 64/14 was measured. The isolated protein was radiolabeled and examined by urea gel analysis for homogeneity. The

TABLE 1. Summary of inhibition experiments of PPACK-reacted, ^{125}I-labeled Lys-plasmin binding to the group A *Streptococcus* strain 64/14 and a schematic depiction of the portion of the native molecule they represent[a]

COMPETITOR		$I_{50\%}$ (μM)
Glu-Plasminogen	Glu1 — 790	> 2.0
Lys-Plasminogen	Lys77 — 790	0.010
Lys-Plasmin	Lys_{77} — 790	0.012
Mini-Plasminogen	Val_{442} — 790	> 1.0
Mini-Plasmin	Val_{442} — 790	> 1.0
LBS-I(K_1-K_3)	Tyr79	> 1.0
LBS-II(K_4)	Val_{354}	> 1.0
Heavy (A) Chain	Lys_{77}	0.046
Light (B) Chain	Val_{561} — 790	> 1.0

[a] Structure and NH_2-amino-terminal residue data were obtained from the work of Sottrup-Jensen et al. (21). Inhibition is expressed as 50% inhibitory values (I_{50}; in micromolar), with Lys-plasmin used as the standard (see text). K indicates kringle domain. Downward arrows indicate the activation cleavage site (Arg-560 to Val-561). The asterisk indicates the plasmin active-site residues (His-602, Asp-645, and Ser-740), from left to right.

labeled material demonstrated a single band on an autoradiograph (Fig. 4A, lane 2) at a position corresponding to that reported for the migration of Lys-plasminogen in this gel system (22). This labeled form of plasminogen was found to bind to the bacteria (Fig. 4B, lane 2). Similarly, the Glu-plasmin generated from Glu-plasminogen in the presence of aprotinin was also capable of binding to the bacteria (Fig. 4B, lane 3). The relative efficiency of unlabeled Glu-plasminogen, Lys-plasminogen, or Lys-plasmin to compete with labeled Lys-plasmin for binding sites on group A *Streptococcus* strain 64/14 was tested. Different concentrations of each of these molecules were mixed with a fixed concentration (1.0×10^{-4} μM) of PPACK-reacted, ^{125}I-labeled Lys-plasmin, and the extent of inhibition of binding of radiolabel was measured as described above. The results of this experiment (Fig. 5) indicate that the inhibition achieved with Lys-plasminogen and Lys-plasmin was identical. These results indicate that the receptor for these ligands is the same and that the affinity for each protein is equivalent.

The possibility that the results presented in Fig. 5 could be accounted for by the conversion of Lys-plasminogen to Lys-plasmin during the reaction was considered. The next series of experiments was designed to determine whether Lys-plasminogen binds to the bacteria without first being activated. These experiments were carried out by monitoring the distribution of Lys-plasminogen, Glu-plasminogen, or Lys-plasmin in the fluid phase and whether they were associated with the bacteria following incubation of the protein with the bacteria. Unlabeled Glu-plasminogen, Lys-plasminogen, or Lys-plasmin was added to a fixed concentration of group A *Streptococcus* strain 64/14 and incubated for 30 min at 37°C. Following this incubation period, the bacteria were pelleted by centrifugation and the supernatants were recovered and monitored for enzymatic activity

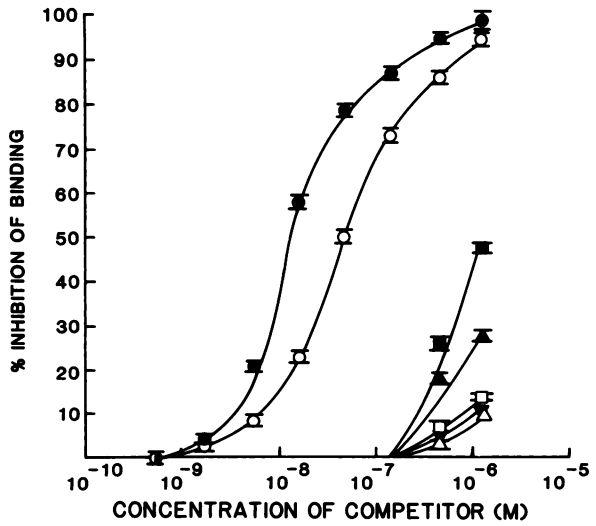

FIG. 3. Inhibition of PPACK-reacted, ^{125}I-labeled Lys-plasmin binding to group A streptococcal plasmin receptor. A constant concentration (1.0×10^{-10} M) of PPACK-reacted, ^{125}I-labeled Lys-plasmin and an increasing concentration of unlabeled competitor molecules (10^{-10} to 10^{-6} M) were mixed with a fixed concentration of *Streptococcus* strain 64/14. Following incubation and washing (see text), the amount of radiolabeled Lys-plasmin bound to the bacterial pellet was determined. The quantity of radioactivity bound in the presence of unlabeled competitor was compared with the radioactivity bound in the absence of inhibitor, and the percent inhibition was calculated. Symbols: ●, Lys-plasmin; ○, heavy chain; ■, LBS-I; □, LBS-II; ▲, light chain; △, mini-plasminogen; ▼, mini-plasmin.

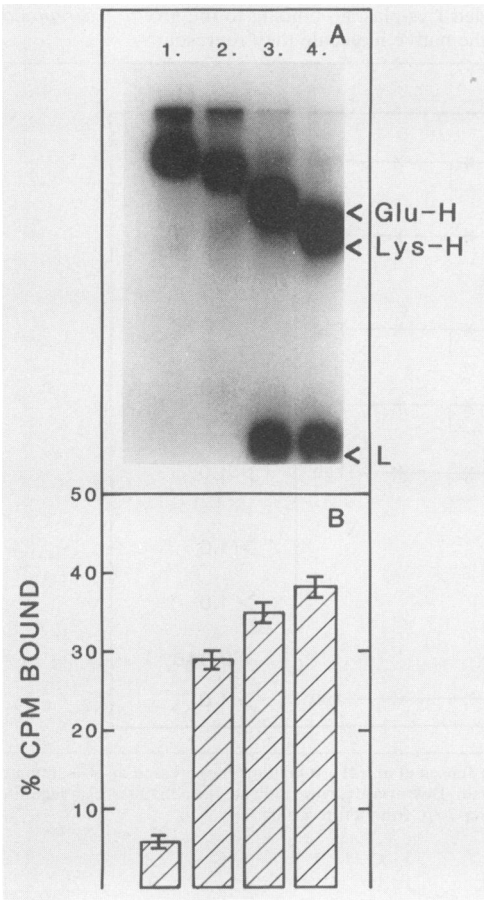

FIG. 4. Binding of ^{125}I-labeled Glu- and Lys-plasmin(ogens). Glu- and Lys-plasmin(ogens) were generated as described in the text. The labeled tracers were then used in direct binding assays with a fixed concentration of *Streptococcus* strain 64/14. (A) Autoradiograph demonstrating the analysis of each reduced ^{125}I-labeled sample on an SDS–6 M urea–12% polyacrylamide gel by autoradiography to verify their molecular form. Abbreviations: Glu-H, Glu heavy chain; Lys-H, Lys heavy chain; L, light chain. (B) Percentage of offered counts per minute bound to bacterial pellets. Lanes: 1, Glu-plasminogen; 2, Lys-plasminogen; 3, Glu-plasmin; 4, Lys-plasmin.

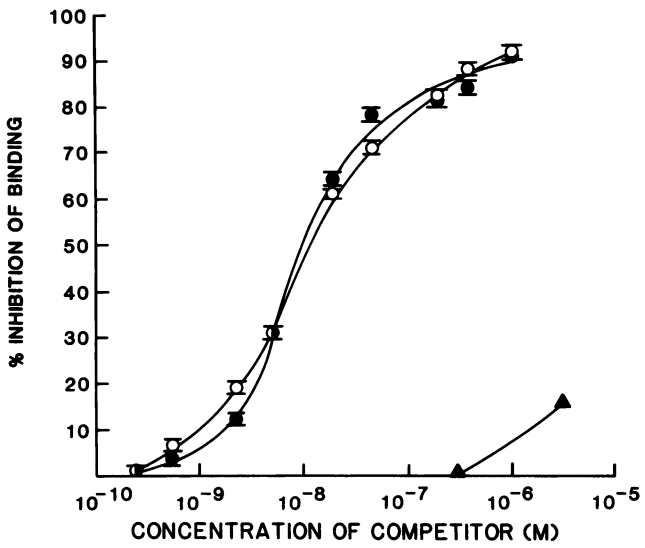

FIG. 5. Inhibition of PPACK-reacted, ^{125}I-labeled Lys-plasmin binding to the streptococcal group A plasmin receptor. A constant concentration (10^{-10} M) of PPACK-reacted, ^{125}I-labeled Lys-plasmin and an increasing concentration range (10^{-10} to 10^{-6} M) of Lys-plasminogen, Glu-plasminogen, or Lys-plasmin were mixed with a fixed concentration of *Streptococcus* strain 64/14. Following incubation and washing (see text), the amount of radiolabeled Lys-plasmin bound to the bacterial pellet was determined. The quantity of radioactivity bound in the presence of unlabeled competitor was compared with the radioactivity bound in the absence of inhibitor, and the percent inhibition was calculated. Symbols: ○, Lys-plasmin; ●, Lys-plasminogen; ▲, Glu-plasminogen.

(Lys-plasmin) or following activation by treatment with excess streptokinase (Glu-plasminogen or Lys-plasminogen), as described above. Following incubation with bacteria and removal of the bacteria by centrifugation, there was no significant Lys-plasmin activity detectable in the bacterium-free supernatant (Table 2). By contrast, over 90% of the enzymatic potential of Glu-plasminogen was detected in the supernatant following activation with streptokinase, while in similar experiments with Lys-plasminogen, less than 10% of the enzymatic potential was measured following activation with streptokinase (Table 2). Because of differences in the efficiency of detection of plasmin activity in the fluid phase compared with detection of its activity when it was bound to bacteria, it was not possible to quantitate accurately the exact percentage of plasmin activity that was bound to the bacteria. However, we have demonstrated previously that once associated with bacteria, the plasmin retains its ability to cleave synthetic chromogenic substrates like S-2251 (14). Consequently, the washed pellets from the absorption reaction were incubated with this synthetic substrate. Bacteria preincubated with Lys-plasmin exhibited significant amidolytic activity (data not shown). Bacteria incubated with Glu-plasminogen exhibited no enzymatic activity, while bacteria incubated with Lys-plasminogen demonstrated a low level of enzyme activity (approximately 15% of that observed in the samples that were preincubated with Lys-plasmin; data not shown). The bacterium-free supernatant of the sample incubated with Lys-plasminogen demonstrated that <10% of the zymogen remained in the supernatant, as detected by measuring the enzymatic activity following activation with streptokinase (Table 2). Taken together,

TABLE 2. Removal of Lys-plasminogen from solution following incubation with bacteria

Reaction mixture	Enzyme activity in bacterium-free supernatant[a]	
	Without activation	Following activation with streptokinase
Glu-plasminogen alone	0	+++
Glu-plasminogen and bacteria	0	+++
Lys-plasminogen alone	0	+++
Lys-plasminogen and bacteria	0	0
Lys-plasmin alone	+++	–
Lys-plasmin and bacteria	0	–

[a] For precise experimental details, see text. Symbols: +++, >90% of the enzymatic potential (i.e., following activation for the zymogen) was added to the reaction mixture; 0, >10% of the enzymatic potential (i.e., following activation for the zymogen) was added to the reaction mixture; –, not relevant.

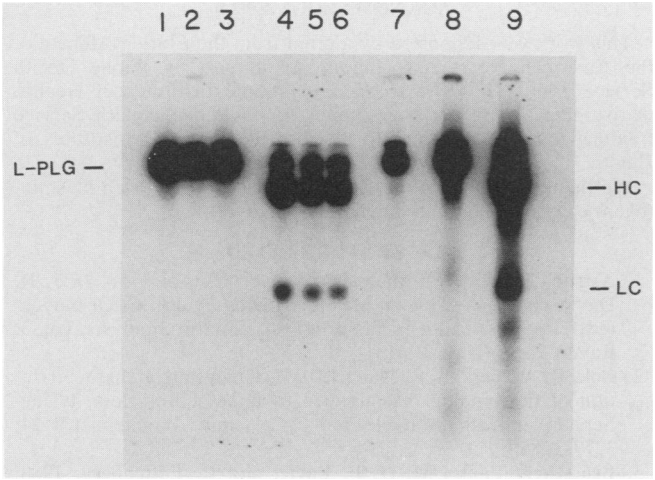

FIG. 6. Characterization of ^{125}I-labeled Lys-plasmin(ogen) species eluted from bacteria. Eluted labeled proteins were analyzed by electrophoresis on an SDS–6 M urea–10% polyacrylamide gel under reduced conditions. Lanes 1, 2, and 3, Labeled proteins from bacteria preincubated with ^{125}I-labeled Lys-plasminogen and eluted with 0.5% SDS, 0.1 M EACA, or 0.5% SDS containing 2% mercaptoethanol, respectively; lanes 4, 5, and 6, the same as lanes 1, 2, and 3, respectively, with the exception that the bound labeled proteins were preincubated with urokinase prior to elution; lane 7, ^{125}I-labeled Lys-plasminogen incubated at 37°C without bacteria for the period of the experiments; lanes 8 and 9, ^{125}I-labeled Lys-plasminogen and Lys-plasmin, respectively. For precise experimental details, see text. Abbreviations: L-PLG, Lys-plasminogen; HC, heavy chain; LC, light chain.

these results indicate that the Lys-plasminogen was removed from the fluid phase without the prior or concomitant activation to Lys-plasmin.

These findings were confirmed in studies in which bacterium-bound, ^{125}I-labeled Lys-plasminogen was eluted from the bacteria and examined by PAGE on 6 M urea gels under reducing conditions. A single protein band corresponding to the enzymatically inactive, single-chain, modified zymogen form of the protein, Lys-plasminogen, was observed on the autoradiograph (Fig. 6). All of these studies demonstrate that Lys-plasminogen can bind to bacteria without first being converted to Lys-plasmin. This indicates that the intact native plasminogen molecule (Glu-plasminogen) does not express structures that are recognized by the bacterial plasmin receptor. However, following a conformational change achieved by either conversion to the Lys-plasminogen form of the zymogen or by activation to plasmin, structures are formed or exposed on the molecule that facilitate interaction with the bacteria.

DISCUSSION

Plasmin is the key component of the mammalian fibrinolytic enzyme system which is responsible for fibrin degradation and intravascular blood clot lysis. Active plasmin, which cleaves fibrin, is derived from the circulating zymogen precursor Glu-plasminogen. Glu-plasminogen is a single-chain glycosylated protein containing 790 amino acids of known sequence with a molecular weight of approximately 92,000 (30, 31). The generation of plasmin from plasminogen is accomplished by proteins known as plasminogen activators. This conversion is brought about by cleavage of a single Arg-560 to Val-561 peptide bond which creates, through conformational changes, a two-chain active plasmin molecule that is held together by disulfide linkages (for a review, see reference 1). The light chain of plasmin has a molecular weight of approximately 25,000 and contains the serine protease active site (19, 31). The heavy chain of plasmin has a molecular weight of approximately 63,000 (19) and contains five homologous triple-loop structures known as kringles (20, 21). An additional conformationally distinct form of plasminogen can be generated when Glu-plasminogen is exposed to plasmin. This removes a 76-amino-acid peptide from the NH_2 terminus, thereby generating Lys-plasminogen (22).

The plasmin(ogen) molecule contains several characteristic lysine-binding sites, one which has a high affinity for the lysine analog EACA (dissociation constant, 9.0 μM) and four or five which have low affinity for EACA (dissociation constant, 5 mM) (15, 16). The high-affinity site has been mapped to kringle domain 1, and one of the lower-affinity sites has been mapped to kringle domain 4 of the plasmin (ogen) molecule (11). These structures are known to participate in the binding of plasmin(ogen) to α_2-antiplasmin (32) and fibrin (22, 33), respectively. It is known that the binding of lysine and lysine analogs to lysine-binding sites on plasmin(ogen) induces conformational changes in the molecule (27). We have shown previously that lysine or α_2-antiplasmin inhibits the binding of plasmin to the group A streptococcal receptor (3, 14), indicating the possible involvement of the high-affinity lysine-binding site in the plasmin-bacterial receptor interaction.

A comparison of our findings with those in studies of the interaction of plasminogen with other naturally occurring plasminogen-binding proteins revealed a number of interesting similarities and contrasts (1, 4, 5, 7). Specific binding to the group A *Streptococcus* strain 64/14 was demonstrated with the heavy chain of plasmin. However, the isolated heavy chain alone was not as efficient a competitor as intact Lys-plasmin was, as evidenced by the nonsuperimposable nature of the heavy chain and Lys-plasmin inhibition curves (Fig. 3). It should be noted that 100% inhibition of binding of Lys-plasmin could be achieved by the addition of high concentrations of heavy chain, but none of the kringle-containing fragments (lysine-binding domains) alone or in combination had any significant inhibitory effects at similar molar concentrations. This finding stresses the importance of the conformation of the entire heavy chain for binding to bacteria. The bacterial binding of plasmin therefore differs from the kind of interaction seen with α_2-antiplasmin, as well as with fibrin and fibrinogen, with which plasmin as well as plasminogen, LBS-I, and LBS-II are known to interact (22, 25, 33).

Consistent with our initial observations (14), there was no significant binding of the native zymogen Glu-plasminogen, while the conformationally altered form of the zymogen, Lys-plasminogen, was found to bind specifically to bacteria (Fig. 4 and Table 2). This form of the zymogen molecule is known to be conformationally distinct from Glu-plasminogen and is similar in conformation to Lys-plasmin (27). The binding of Lys-plasminogen to the group A streptococcal receptor is therefore dependent on a specific conformation, probably of the heavy chain. It is of interest that despite the observed inhibitory effects of lysine or lysine analogs on the binding of plasmin to its bacterial receptor (3), there was no significant reactivity with any of the isolated plasminogen domains containing either high- or low-affinity lysine-binding sites (Table 1). These results need to be interpreted cautiously, since the affinity of isolated kringle domains for

lysine-Sepharose does not appear to be as high as that observed in studies done with the intact molecule (2, 34). Based on the inhibitory effects of the isolated heavy chain shown in Fig. 3 and our previous demonstration that bacterial plasmin binding is inhibitable by α_2-antiplasmin (14) or lysine or lysine analogs (3), a role for the high-affinity binding site on the intact plasminogen molecule cannot be completely excluded in the interaction of Lys-plasminogen or plasmin with the bacterial receptor.

Two other plasmin(ogen)-binding proteins, thrombospondin (12, 28) and histidine-rich glycoprotein (8, 13), have been shown to react with the high- and low-affinity lysine-binding sites on the heavy chain of Glu-plasminogen. These proteins display differences in binding profiles; however, neither of the molecules displays the marked difference in recognition of Glu-plasminogen and Lys-plasminogen or Lys-plasmin that we documented for the bacterial plasmin receptor.

Of particular relevance to this study is the interaction of plasmin(ogen) with the well-characterized streptococcal plasminogen activator streptokinase isolated from group C streptococci (6, 26). This secreted streptococcal protein is known to bind rapidly to Glu-plasminogen, Lys-plasminogen, and Lys-plasmin (rate constant, $5.4 \times 10^7\ M^{-1}\ s^{-1}$), forming a 1:1 stoichiometric complex with an estimated dissociation constant of 5×10^{-11} M (5). This interaction occurs via an interaction with the light chain (23). The interaction with the group A streptococcal plasmin receptor is distinct from the interaction with group C streptokinase, in that it does not recognize the Glu-plasminogen molecule and demonstrates no reactivity with the isolated light chain of plasmin. Furthermore, the plasmin(ogen)-streptokinase complex cannot be dissociated by lysine or lysine analogs (G. O. Von Mering, M. D. P. Boyle, and R. Lottenberg, Clin. Res. **36**:464a, 1988), while the interaction of plasmin with a group A streptococcus is completely reversible by lysine or lysine analogs (3).

Taken together, our results indicate that the group A streptococcal plasmin receptor binds in a unique manner to both plasmin and Lys-plasminogen. The predominant interaction is via determinants that are present on the intact heavy chain. These structures are present in their optimal binding configurations on the intact plasmin molecule and on the modified zymogen Lys-plasminogen. The observations that plasmin bound to bacteria retains its enzymatic activity for both small synthetic substrates and for fibrin (14) are consistent with the observation that the light chain is not involved in binding. The failure of α_2-antiplasmin to regulate the bound enzyme suggests that the required interaction between α_2-antiplasmin and plasmin is directly or indirectly inhibited. This may occur because one of the recognition sites for α_2-antiplasmin in the kringle 1 domain of the heavy chain of plasmin may not be accessible when plasmin is bound to a *Streptococcus* isolate.

The characteristics of the interaction of human plasmin with group A *Streptococcus* strain 64/14 described in this report indicate that the bacteria can capture a potent protease activity that cannot be regulated by the primary physiological inhibitor of plasmin, α_2-antiplasmin. This group A *Streptococcus* strain also secretes a plasminogen activator, and consequently, in the presence of plasminogen, the bacterium has the potential to both generate plasmin and bind the active enzyme to its surface (L. E. DesJardin, M. D. P. Boyle, and R. Lottenberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, B-169, p. 57). The importance of this selective receptor to the infectious disease process of receptor-positive bacteria remains to be established.

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