ISOLATION OF A LOW MOLECULAR WEIGHT FORM OF PLASMINOGEN.*

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SUMMARY: Treatment of sheep plasminogen with sheep plasmin results in the cleavage of at least two peptides from sheep plasminogen. One peptide of molecular weight 6,000-8,000 is rapidly liberated and second peptide, of molecular weight 29,000-33,000, is more slowly cleaved. The remainder of the molecule possesses a molecular weight of 50,000-55,000 and represents a fully activatible plasminogen. Upon treatment of this low molecular weight plasminogen with urokinase, esterolytic and fibrinolytic activity result. The plasmin obtained consists of two disulfide linked polypeptide chains of molecular weights approximately 30,000 and 24,000. Electrophoretically similar plasmin chains are produced upon activation of native sheep plasminogen with urokinase.

Recent studies on human (1-4) and rabbit plasminogen (5,6) demonstrate that an altered plasminogen, of molecular weight 6,000-8,000 less than the native plasminogen, is generated during the course of their activation to plasmin by urokinase. In each case, this peptide(s) is removed from the amino terminus of plasminogen, generating a new plasminogen with drastically altered activation (6,7) and conformational (8,9) properties. During the course of our studies, we have discovered that in addition to this small peptide, a much larger peptide is also released from sheep plasminogen. This results in a much smaller plasminogen molecule, which is the subject of this report.

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MATERIALS AND METHODS

Proteins. Sheep plasminogen was isolated in a single step from whole phenylmethyl sulfonyl fluoride treated citrated sheep plasma by our gradient elution modification (10) of the Deutsch and Mertz affinity chromatography method (11). For studies reported here, fraction 2 sheep plasminogen was utilized.

Urokinase, prepared by tissue culture techniques from human embryonic kidney cells, was a generous gift of Dr. Grant Barlow.

Urokinase free-sheep plasmin was prepared by activation of sheep plasminogen by insolubilized urokinase, as we have described for the rabbit system (6). Since the activation times of rabbit and sheep plasminogen differ and also depend upon the quality and efficiency of coupling of urokinase, we follow the generation of TosArgOMe activity as a monitor of the rate of activation of plasminogen with Sepharose-urokinase and terminate the activation at peak TosArgOMe activity. The concentration of active sheep plasmin was determined by the p-nitrophenyl-p'-guanidinobenzoate burst assay.

The low molecular weight form of sheep plasminogen was isolated by affinity chromatography after preparation by plasminolysis of native sheep plasminogen. In particular 2 ml of a solution of 14.3 mg/ml of sheep plasminogen in 0.05 M tris·HCl, 0.1 M L-lysine, pH 8.0 was treated with 0.5 ml of urokinase free sheep plasmin (0.4-1.0 mg/ml of active plasmin) in the same buffer. The solution was allowed to incubate at 30° for 4 hours. This solution was then diluted to 100 ml with 0.3 M phosphate, pH 8.0, and passed over the original Sepharose 4-B-L-lysine column as used for the purification of sheep plasminogen. After washing the column with 100 ml of 0.3 M phosphate, elution with a similar ε-amino caproic acid gradient (limit solution 0.02 M ε-amino

caproic acid) as previously described (10) results in resolution of three well resolved peaks. The first is altered sheep plasminogen; the second is a large peptide and the third is native sheep plasminogen. The sheep plasmin originally added is degraded during the incubation and thus passes unretarded through the column.

Analytical techniques. Sodium dodecyl sulfate gels, in the presence and absence of β-mercaptoethanol, were essentially performed as described by Weber and Osborn (12). The amounts of polyacrylamide utilized were varied in different experiments. Human fibrin plates, containing essentially no human plasminogen, were purchased from Hyland. Plasmin assays, utilizing these plates, were performed as suggested by the manufacturer. TosArgOMe assays were performed by the potentiometric method. Our particular procedure has been described (13).

RESULTS AND DISCUSSION

Figure 1 shows a reduced sodium dodecyl sulfate gel electrophoretic analysis of the time course of digestion of sheep plasminogen with sheep plasmin under conditions described in Methods. At very short time periods (<11 min) the native sheep plasminogen (SPg$\underline{a}$) is cleaved to a slightly smaller species (SPg$\underline{b}$). At longer times, Pg$\underline{b}$ is cleaved to two large peptides, SPg$\underline{c}$ and P'. No difference in behavior was noted on unreduced gels, suggesting that none of the components are disulfide linked. Calibrated sodium dodecyl sulfate gels allowed estimates of molecular weights of each component liberated and these values are listed in Table I. At no time during the time studies reported in Figure 1 did plasmin activity, other than what was initially added, appear. Thus, none of the cleavages catalyzed by plasmin lead to generation of other plasmin molecules. However, addition of the acti-

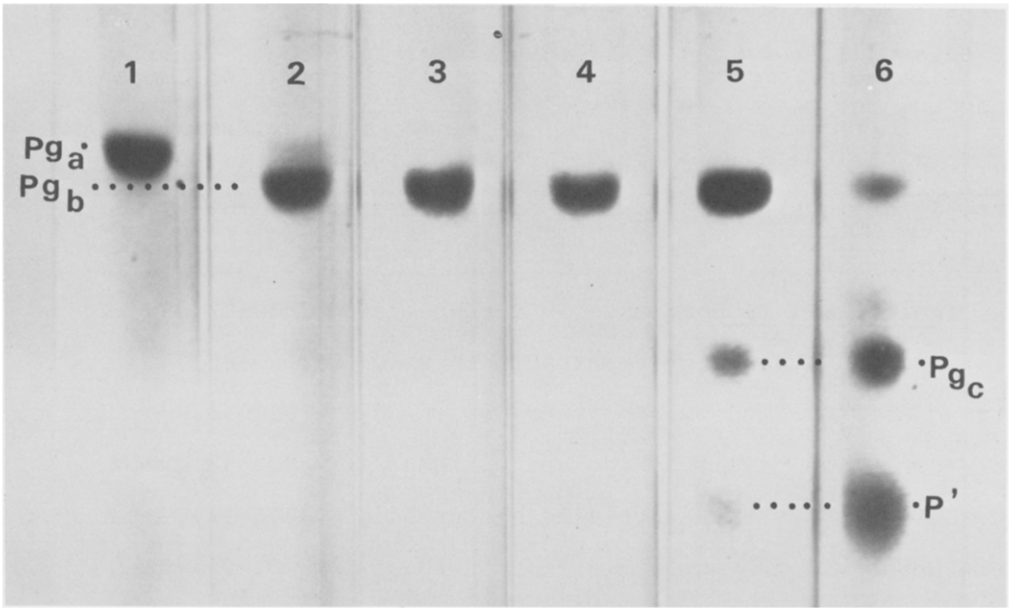

Figure 1. Effect of sheep plasmin on sheep plasminogen as monitored by reduced sodium dodecyl sulfate gel electrophoresis on 5% polyacrylamide gels. The conditions are as described in Methods. Gel 1, native sheep plasminogen incubated separately overnight. Native sheep plasminogen (SPga) was then treated with plasmin for times of, gel 2, -2 minutes; gel 3, -11 minutes; gel 4, -21 minutes; gel 5, -120 minutes; gel 6, -240 minutes.

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vator urokinase to the material represented by the 2 hour point in Figure 1, led to a rapid generation of plasmin activity and the disappearance of the Pgc band.

The above results suggested that the Pgc band was a fully functioning plasminogen. This was confirmed by the isolation and characterization of this material by affinity chromatography. In this experiment, plasmin and SPga were incubated for four hours under conditions similar to Figure 1. The reaction mixture was then treated as described in Methods. Figure 2 shows gel analysis of the material obtained. Also in Figure 2 we show the effect of treatment of SPga, SPgc and P' with urokinase at time periods

Table I

Molecular Weights of Sheep Plasminogen Activation Components as Observed on Reduced Sodium Dodecyl Sulfate Gels

Component	Molecular Weight
SPg$\underline{a}$[a]	87,000-92,000
SPg$\underline{b}$[a]	80,000-85,000
SPg$\underline{c}$[a]	50,000-55,000
P'[a]	29,000-33,000
SPmH[b]	29,000-32,000
SPmL[c]	23,000-26,000

[a]Abbreviations are defined in the text.

[b]Refers to the sheep plasmin heavy chain.

[c]Refers to the sheep plasmin light chain.

whereby full plasmin activity is obtained. SPg$\underline{a}$ is converted into 2 bands of approximate molecular weights of 30,000 and 24,000. The 30,000 mobility band consists of P' and the sheep plasmin heavy chain. The 24,000 band consists of the sheep plasmin light chain. Unreduced sodium dodecyl sulfate gels demonstrate that the plasmin heavy and light chains are disulfide linked. Further, this figure shows that SPg$\underline{c}$ is also converted by urokinase into the same two molecular weight bands as obtained for SPg$\underline{a}$. These two bands represent the plasmin heavy and light chains (the peptide, P', has been removed by the affinity chromatography procedure). Accompanying studies on unreduced gels show that

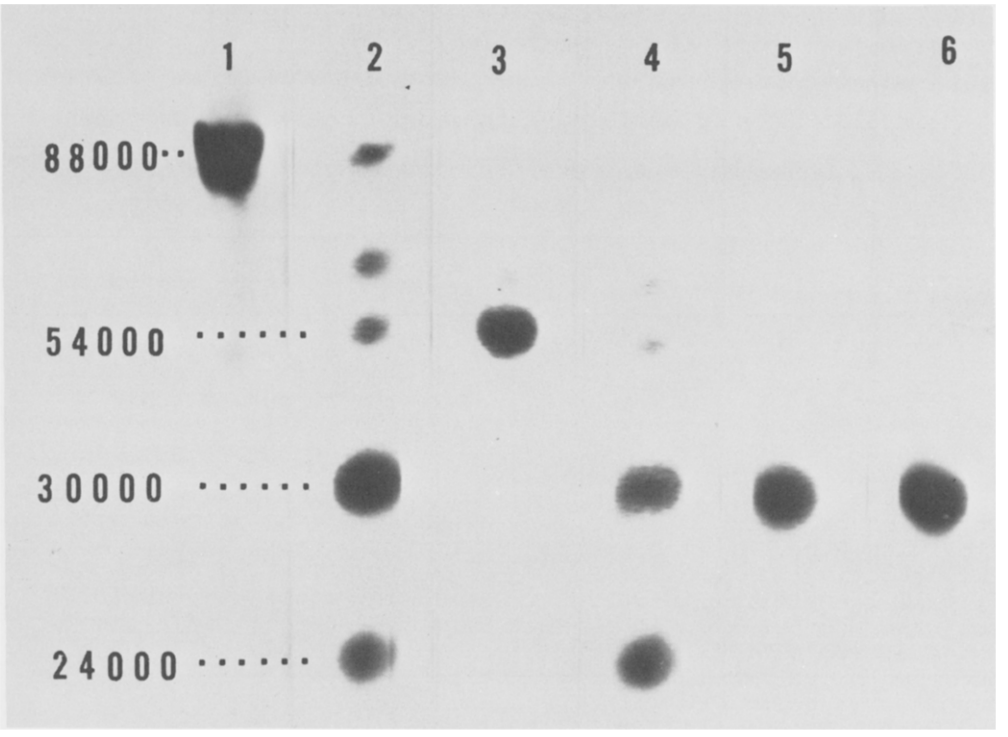

Figure 2. Reduced sodium dodecyl sulfate gels of purified sheep plasminogens and their activation components. Gel 1, sheep plasminogen a; gel 2, the material in gel 1 treated with urokinase until maximal plasmin activity occurred; gel 3, sheep plasminogen c purified by affinity chromatography; gel 4, the material in gel 3 treated with urokinase until maximal plasmin activity occurred; gel 5, the purified large peptide released from sheep plasminogen a upon treatment with plasmin; gel 6, the material in gel 5 treated with urokinase.

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these two plasmin peptide chains are disulfide linked. As is also shown in this figure, urokinase is without effect on P'.

A fibrin plate assay on the activation product SPgc is shown in Figure 3. A ring of lysis is obtained only for urokinase treated SPgc, thereby establishing that plasmin is obtained from activation of SPgc. Identical results were obtained upon substituting SPga for SPgc. This shows that the plasmin obtained from either plasminogen is equivalent in activity. Similar conclusions are derived from esterase assays utilizing TosArgOMe. Here, the peak

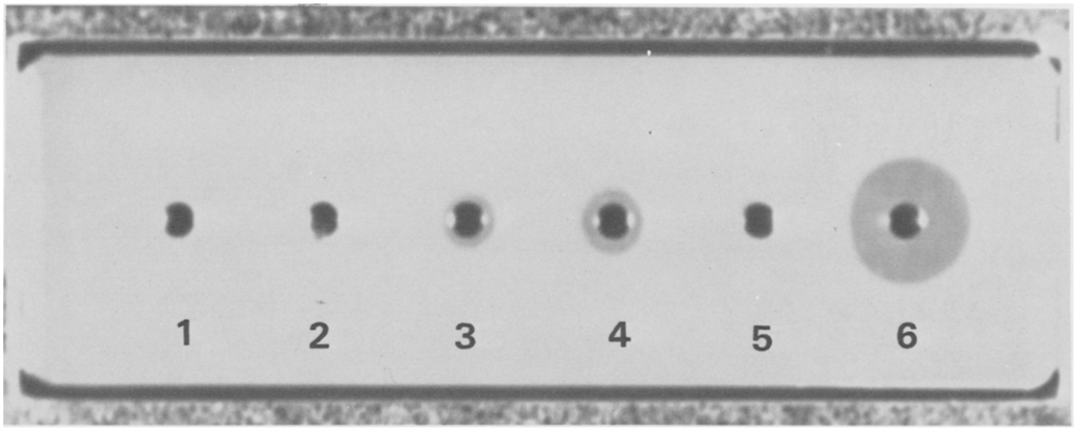

Figure 3. Fibrin plate assay of sheep plasminogen c and its activation products. Here 0.1 ml of SPgc (0.2 mg/ml) was activated with 0.005 ml (110) units of urokinase for 2 hours. A similar activation was attempted with P'. Well 1, 0.005 ml of P'; well 2, material in well 1 treated with urokinase; well 3, urokinase alone tested at zero time; well 4, urokinase alone tested at 120 minutes of incubation; well 5, 0.005 ml of SPgc incubated alone for 120 minutes; well 6, 0.005 ml of SPgc incubated as described with urokinase.

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esterase activities obtained by urokinase treatment of SPga and SPgc are 1.3 and 1.0 μmoles TosArgOMe cleaved min^{-1} nmole plasminogen activated. These values are not maximal since the TosArgOMe concentration is not saturating and the concentration of active plasmin sites has not been measured at this stage of the investigation.

These studies show that approximately 1/3 of the SPga molecule can be released without apparently affecting the ability of plasminogen to be activated to plasmin. This situation is ideal for investigating structure-function relationships in plasminogen. These studies, as well as studies derived at elucidating a role for the released large peptide, are currently underway.

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