THE MECHANISM OF ACTIVATION OF RABBIT PLASMINOGEN BY UROKINASE. LACK OF A PREACTIVATION PEPTIDE.*

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Received August 9,1974

SUMMARY: We have obtained direct evidence which we interpret to prove that an amino terminal peptide need not be released from rabbit plasminogen prior to its conversion to plasmin by urokinase. The single chain plasminogen molecule possesses an amino terminal amino acid sequence of NH_2-glu-pro-leu-asp-asp. When this plasminogen is activated to plasmin by urokinase in the presence of the Kunitz bovine trypsin-plasmin-kallikrein inhibitor (BTI), a two chain disulfide linked molecule of plasmin is obtained. The heavy chain of this plasmin is directly derived from the original amino terminus of plasminogen since it possesses the identical amino terminal sequence as does native plasminogen. When the same plasminogen activation is carried out in the absence of BTI, the heavy chain of the plasmin obtained has a molecular weight of 6,000-8,000 less than the heavy chain of the plasmin obtained in the presence of this inhibitor. In addition, the heavy chain of this latter plasmin has an amino terminal sequence which differs from the original native plasminogen. These data show, in agreement with others, that the activation of plasminogen by urokinase is accompanied by the loss of an amino terminal peptide from plasminogen but also show, in contrast to the human plasminogen system, that cleavage of the internal peptide bond, leading to plasmin formation, can occur without cleavage of the amino terminal peptide.

It is now generally accepted that at least two proteolytic cleavages occur as a final consequence of the activation of plasminogen to plasmin. One cleavage results in the loss of a small peptide (molecular weight 6,000-8,000) from the amino terminal end of the single chain plasminogen molecule (1-4) and another

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*These studies were supported by Grants HL-13423 and HL-15747 from the N.I.H. and a grant-in-aid from the American and Indiana Heart Associations (to F.J.C.) as well as Grant HL-16150 from N.I.H. (to K.G.M.). F.J.C. is a Career Development Awardee from the N.I.H. (HL-70717) and K.G.M. is an established investigator of the A.H.A.
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cleavage, in the interior of the plasminogen molecule, results in the formation of the well known two chain, disulfide-linked plasmin molecule, originally described by Robbins' laboratory (5). Recently, the results of several investigations (3,4) were interpreted to suggest that the release of the above described amino terminal peptide was a requisite first step in the activation mechanism of plasminogen. In fact, for this reason, this peptide has been termed a "preactivation peptide" (4). The removal of this peptide was explained to result in a lower molecular weight, conformationally altered plasminogen (altered plasminogen) which became activated to plasmin (6). This two-chain plasmin molecule contains a heavy chain derived from the amino terminal portion of the altered plasminogen and a light chain derived from the carboxyl terminal portion of plasminogen (5).

Although our studies agreed with the observations that at least two proteolytic cleavages accompanied the activation of plasminogen to plasmin, we felt that the postulation of release of a pre-activation peptide as a necessary first step was based on indirect evidence. In our studies on the activation of rabbit plasminogen by urokinase, we have isolated, in high yield, a heavy chain of plasmin containing the intact amino terminal sequence of the original native plasminogen. Some of the important data on this point, which is summarized in this communication, provides compelling evidence against any mechanism of plasminogen activation which postulates, as a first step, the cleavage of this amino terminal peptide from plasminogen.

MATERIALS AND METHODS

Proteins. The plasminogens used in this study were purified by gradient elution from affinity chromatography, as we have previously described (7). In each case affinity fraction 2 was

used as the source of plasminogen. Urokinase was purchased from Calbiochem and the Kunitz inhibitor was purified from bovine lung as described by Kassell (8) except that we used Sepharose-4B-trypsin as the affinity chromatography resin.

Plasminogen activation. The following stock solutions were utilized in the activation of plasminogen: plasminogen (9.35 mg/ml) in 0.05 M tris·HCl - 0.1 M L-lysine, pH 8.0; Kunitz inhibitor (BTI) (9.96 mg/ml) in the same buffer and urokinase (5893 Plough units/ml), also in the same buffer. The activation in the absence of BTI was carried out at 22° for 30 minutes by mixing 4.3 ml of the stock plasminogen solution, 2.1 ml of the tris-lysine buffer and 1.42 ml of the stock urokinase. The activation in the presence of BTI was carried out at 22° for 75 minutes by mixing 4.3 ml of the stock plasminogen, 2.1 ml of the stock BTI solution and 1.42 ml of the stock urokinase.

Isolation of the plasmin polypeptide chains. At the conclusion of the activation incubation in either case, the pH of the solution was lowered to pH 2 by addition of HCl and the mixture was lyophilized. The powder was dissolved in 3 ml of 6 N guanidine hydrochloride (Heico, Inc.) - 0.05 M tris·HCl and the pH was adjusted to 8.6. At this stage 1.1 mmoles of β-mercaptoethanol was added and the mixture flushed with N_2. This solution was allowed to incubate for 12 hours at 37°. At this time 1.3 ml of a stock solution containing 1.0 mmoles/ml of recrystallized iodoacetate in 6 M guanidine hydrochloride - 0.05 M tris·HCl, pH 8.6 was added. The pH was maintained at 8.6 by addition of 4 N NaOH for 15 minutes at 22°C. Excess iodoacetate was then destroyed by addition of a little β-mercaptoethanol. This solution was then applied to a 2 cm x 115 cm column of Sephadex G-150 in 4 M guanidine hydrochloride. The plasmin heavy and light chains were easily resolved by this column.

Quantitative amino terminal end group analysis of the dialyzed and lyophilized heavy and light chains resolved as a consequence of either mode of activation of plasminogen were subjected to the quantitative procedure of Stark (9). The concentration of material taken for these analyses was determined by amino acid analysis of an aliquot of the cyclization mixture.

Amino terminal amino acid sequences of plasminogen as well as the plasmin heavy and light chains were determined on a Beckman Model 890 B Sequencer. The methods used are essentially as previously described (10).

Sodium dodecyl sulfate gel electrophoresis was performed according to the procedure of Weber and Osborn using 5% acrylamide gels (11).

RESULTS

It had previously been demonstrated that BTI was a much more potent inhibitor of plasmin than of urokinase (12). We have taken advantage of these selective inhibitory properties of this inhibitor to completely inactivate the plasmin produced during the activation of plasminogen by urokinase while minimizing the inhibition of urokinase. Under the conditions described in "Methods" the activation rate of rabbit plasminogen was only slightly inhibited by BTI and the generated plasmin was rapidly and completely inactivated. The sodium dodecyl sulfate-mercaptoethanol gel behavior of the activation mixtures described in "Methods" are shown in Figure 1. In the absence of BTI, a disulfide linked heavy and light plasmin chain can be demonstrated, as expected, for rabbit plasmin (13). This heavy chain appears at the shortest time in which the greatest plasmin activity is noted and remains stable for a relatively long period. The amino

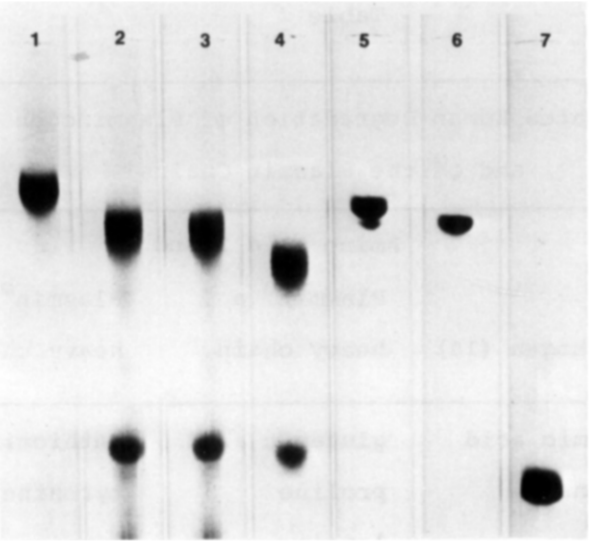

Figure 1. Sodium dodecyl sulfate-mercaptoethanol gels of the materials obtained in this study: 1, rabbit plasminogen; 2, rabbit plasmin obtained in the presence of BTI (plasmin a); 3, as in 2. except that the urokinase level is increased 3 fold; 4, rabbit plasmin obtained in the absence of BTI (plasmin b); 5, purified rabbit plasmin a heavy chain; 6, purified rabbit plasmin b heavy chain; 7, rabbit plasmin light chain. Gels 1, 2, 3 and 4 were run at the same time and can be compared with each other. Gels 5, 6 and 7 were also run at the same time and can also be compared with each other but not with gels 1-4. In addition, Gels 5, 6 and 7 contained 6 M urea.

terminal sequence for each chain, isolated in 80-90% yield, is shown in Table I. Although it is well known that the plasmin heavy chain is derived from the amino terminus of plasminogen, it is clear that no similarity exists when the sequences of the original plasminogen and the plasmin heavy chain are compared (Table I). This is taken to demonstrate that an amino terminal peptide is removed from plasminogen (or a different heavy chain) at some point during the activation of plasminogen. On the other hand, the amino terminal amino acid sequence of the purified heavy chain, isolated in approximately 80% yield, from the plasmin obtained from the activation of plasminogen in the presence of BTI, possesses an identical amino terminal sequence

Table I

Automated Edman Degradation of Plasminogen and of the Plasmin Chains

Residue number	Amino acid found in Plasminogen (10)	Plasmin[a] a heavy chain	Plasmin[b] b heavy chain	Light chain
1	glutamic acid	glutamic acid	methionine	valine
2	proline	proline	tyrosine	valine
3	leucine	leucine	leucine	gly
4	aspartic acid	aspartic acid	—	gly
5	aspartic acid	aspartic acid	glutamic acid	—
6	tyrosine	tyrosine	—	val

[a]Refers to the plasmin obtained by activation of plasminogen in the presence of BTI.

[b]Refers to the plasmin obtained by activation of plasminogen in the absence of BTI.

as does the original plasminogen (Table I). These results conclusively demonstrate that the amino terminal peptide need not be cleaved from plasminogen prior to the cleavage of the internal peptide bond, leading to the two chain plasmin structure. Further, it can be observed from the gels that a molecular weight difference exists in the heavy chains of the two separate activation mixtures. Careful sedimentation equilibrium analyses indicate that the heavy chain isolated from the activation in BTI has a molecular weight of 6,000-8,000 greater than the heavy plasmin chain obtained from the activation of plasminogen in the absence of BTI.

Finally, since the results of the activation of plasminogen in the presence of BTI are so important to the activation mechanism of plasminogen, we reconfirmed the presence of glutamic acid on the amino terminus of the isolated heavy chain by the quantitative Stark procedure. Here we obtain 0.9 moles of glutamic acid per mole of heavy chain. This result clearly fortifies the sequenator results and establishes the presence of glutamic acid as the amino terminus of the heavy chain.

DISCUSSION

The results presented in this communication show that it is possible to isolate, in high yield, a heavy chain from the two chain plasmin molecule containing the intact amino terminal sequence of the original plasminogen. This conclusively demonstrates that urokinase can cleave the internal peptide bond in plasminogen to yield the two chain plasmin structure without cleaving the peptide from the amino terminus of either native plasminogen or the plasmin heavy chain. It is of interest to determine whether urokinase is even at all capable of cleaving this amino terminal peptide. In this regard, we have increased the the levels of urokinase 3 fold in the activation mixture (Fig. 1) in the presence of the same levels of BTI and have obtained the same glutamic acid amino terminal heavy chain. Unless BTI has in some unknown manner affected the system, a logical explanation of this experiment is that urokinase does not cleave the amino terminal peptide from plasminogen and would implicate the plasmin generated during the activation process as the species responsible for removal of the peptide. This argument might be extended to include the possibility that plasmin and not urokinase also cleaves the amino terminal peptide from the glu-plasmin heavy chain. However, the possibility that the BTI bound to the plasmin blocks

the accessibility of this amino terminal peptide from cleavage by urokinase cannot be excluded. Although some of our unpublished data argue against this latter point, the matter is still under investigation. In any event, this does not influence the conclusion that the amino terminal free plasminogen is not an obligatory intermediate in the rabbit plasminogen activation mechanism.

Finally, it should be pointed out that although all these studies have been carried out with rabbit plasminogen, we have obtained similar gel electrophoretic behavior with human plasminogen in the presence and absence of BTI.

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