DIRECT EVIDENCE FOR THE GENERATION OF AN ACTIVE SITE IN THE PLASMINOGEN MOIETY OF THE STREPTOKINASE-HUMAN PLASMINOGEN ACTIVATOR COMPLEX

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SUMMARY: A plasmin active site titrant, p-nitrophenyl p-(amidinothiomethyl)benzoate (AmSMeBzoNph), has been prepared with a radioactive label in the acid moiety and utilized to directly demonstrate the generation of an active site in plasminogen upon incubation with streptokinase to form the human plasminogen activator complex. When a 1:1 stoichiometric complex of streptokinase and human plasminogen are incubated for 2 minutes in the presence of a 10 fold excess of [^{14}C]-AmSMeBzoNph, 0.7-0.8 moles of p-nitrophenol are released per mole of plasminogen added and 0.7-0.8 moles of the [^{14}C]-substrate are covalently incorporated into the complex. Sodium dodecyl sulfate polyacrylamide gel electrophoresis demonstrated that only streptokinase and human plasminogen were present and all the radioactivity was found in the plasminogen band. When a 1:1 stoichiometric complex of streptokinase and human plasminogen are incubated for 2 minutes in the presence of a 10 fold excess of unlabeled p-nitrophenyl-p'-guanidino benzoate (GdnBzoNph), followed by the same amount of [^{14}C]-AmSMeBzoNph, no label is incorporated into the complex. This evidence supports the contention that an active site is developed in human plasminogen upon complex formation with streptokinase.

Some key studies (1,2,3) in the last few years have shown that the formation of human plasminogen activator from streptokinase (SK) human plasminogen (HPg) can be described by the following series of reactions:

$$SK + HPg \rightleftharpoons SK \cdot HPg \longrightarrow (SK \cdot HPg)' \longrightarrow (SK^{*} \cdot HPm)'$$

Here the proenzyme, HPg and the inactive bacterial protein, SK interact to form a 1:1 complex. This complex undergoes a rearrangement to form an altered complex (SK·HPg)', which now possesses an active site. This complex is called an activator

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complex since it can activate the single chain human plasminogen molecule to the two chain protease, plasmin (HPm). Under conditions where there is no excess plasminogen to activate, the (SK•HPg)' complex can activate the plasminogen within this complex to plasmin, yielding an (SK^*•HPm)' complex. This complex, which also possesses a proteolytically altered streptokinase (SK^*), is also capable of activating human plasminogen. It is not clear exactly which complex is the activator complex in the presence of excess plasminogen and it is also not clear whether the SK is intact in the (SK•HPg)' complex.

Several studies throughout the years (4,5) demonstrated that the activator active site in the (SK^*•HPm)' complex was the same active site that was found in plasmin. Further, it has been proposed, by indirect means, that the activator active site in the (SK•HPg)' complex was present in the HPg moiety and was the same active site that would ultimately form in plasmin (2,4,6). These concepts were recently challenged by Taylor and Beisswenger (7) who apparently demonstrated that the activator active site resided in the altered streptokinase portion of the activator complex.

Due to this controversy and due to the fact that the presence of an active site in the Pg portion of the activator complex has never been directly demonstrated, we undertook a study in which we attempted to solve these problems. This communication is a result of these investigations.

Materials and Methods - Purified human plasminogen was prepared as previously described (8) and purified streptokinase was donated to us by Dr. Hugo Nihlén.

The compound [^{14}C]-AmSMeBzoNph was synthesized by coupling [^{14}C]-thiourea to p-nitrophenyl p'-bromomethyl benzoate. The

latter compound was synthesized exactly according to Wang and Shaw (9). A quantity of 53.2 mg of unlabeled thiourea and 3.82 mg of [^{14}C]-thiourea (0.5 mc) was added to 2 ml of ethyl acetate. The thiourea is poorly soluble in this reagent and warming to 60^{o} was necessary to improve the solubility. After the solution cooled to room temperature, 168 mg of p-nitrophenyl p'-bromomethyl benzoate was added and the reaction was stirred at room temperature for 3 days. During this time the thiourea becomes soluble and the product precipitates. The precipitate was filtered and dried under a water aspirator vacuum. The final yield was 87% and had a melting point of 216-218^{o} after recrystallization from absolute ethanol. The specific activity was 2.4 x 10^{12} dpm/mole. Analysis of a non-labeled sample, synthesized in the same manner gave the following results: C; theory 43.7%, found 44.0%: H; theory 3.4%, found 3.63%: O; theory 15.5%, found 15.86%: N; theory 10.2%, found 10.3%.

In order to determine whether this compound would react with the active site present in the plasminogen activator complex, 0.6 ml of a solution containing 3.4 x 10^{-5} mmoles of HPg in the presence of 4 x 10^{-4} mmoles of [^{14}C]-AmSMeBzoNph was added to 0.2 ml of a solution containing 3.5 x 10^{-5} mmoles of SK. All components were dissolved in 0.05 M tris·HCl - 0.01 M L-lysine, pH 8.0 at 30^{o}, except the [^{14}C]-AmSMeBzoNph was prepared as a 0.01 M stock solution in dimethyl formamide. This solution was allowed to incubate at room temperature for 2 minutes. An aliquot of this solution was diluted to 1 ml in 0.05 M veronal, pH 8.5 and the optical density at 412 nm was determined in order to obtain an estimate of the amount of p-nitrophenolate released. The remainder of the solution was dialyzed against 4 M guanidine hydrochloride, pH 3.0 in order to remove any noncovalently bound

substrate. At the conclusion of the dialysis, the volume of material in the dialysis sac was measured and an aliquot removed for liquid scintillation counting to determine the amount of substrate covalently bound to the complex. Subsequent to this, another aliquot was dialyzed against sodium dodecyl sulfate (SDS) electrophoresis buffer in the absence of mercaptoethanol and SDS gel electrophoresis was performed in unreduced gels. Duplicate gels were run and one gel was stained for protein and the other gel was sliced for radioactivity determinations. This experiment allowed us to determine whether the radioactivity was present in the band corresponding to plasminogen or streptokinase. In order to determine whether all the initial plasminogen was present as plasminogen or plasmin, we performed SDS gel analysis in reduced gel systems. However, these gels cannot be counted for radioactivity since mercaptoethanol causes a rapid deacylation of the substrate from the protein by a transesterification process.

Results - The amount of p-nitrophenolate released from [^{14}C]-AmSMeBzoNph when 1:1 ratios of human plasminogen and streptokinase are incubated in the presence of an excess of this compound is in the range of 0.7-0.8 moles per mole of plasminogen initially added. Additionally, we find that 0.7-0.8 moles of radioactivity are covalently incorporated per mole of plasminogen into the plasminogen activator complex. Assays at this stage show that no plasminogen activator activity exists in the complex after this treatment, whereas high levels of activator activity exist if the same experiments are performed in the absence of [^{14}C]-AmSMeBzoNph. These results show that the active site of the activator complex must be covalently modified.

In order to determine the moiety of the complex which now

possesses the radioactivity we performed sodium dodecyl sulfate gel electrophoresis experiments on the radioactive complex. These gels are shown in Figure 1. If no reducing agents are

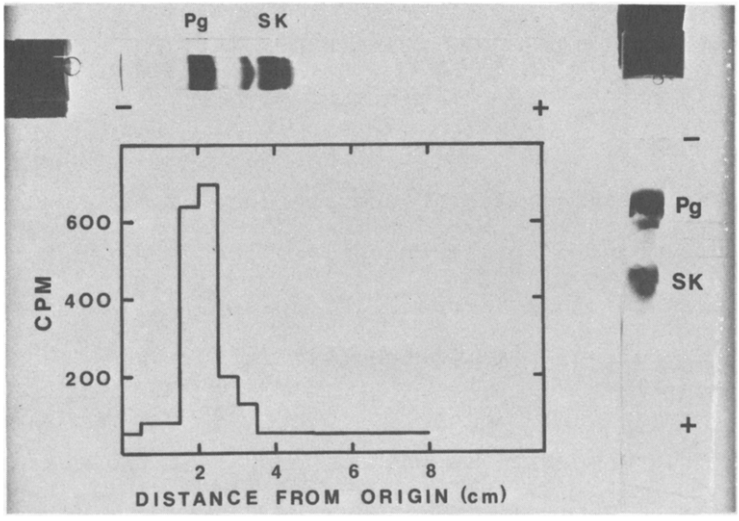

Figure 1. The acylation of human plasminogen in the presence of stoichiometric levels of streptokinase by [^{14}C]-AmSMeBzoNph. A duplicate unreduced sodium dodecyl sulfate gel to that shown here was sliced and the radioactivity eluted from the gel. The radioactivity level determined for each slice is alligned with the gel. Positive identification of the radioactive band as plasminogen (and not plasmin) was accomplished by similar gels analyzed in the presence of mercaptoethanol. This gel is shown on the extreme right of the Figure.

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added, all the radioactivity corresponds to the band with the molecular weight of plasminogen or plasmin. This is also obvious from Figure 1. Reduced sodium dodecyl sulfate gels can distinguish between plasminogen and plasmin and these are also shown in Figure 1. Again, only plasminogen and streptokinase are

present in this case. However, no radioactivity was present in the plasminogen since mercaptoethanol removes the covalent label from the protein by a transesterification reaction. However, it is important to note that undectable levels of plasmin are present in these latter gels. Therefore, all the radioactivity was initially present in plasminogen.

Additionally, if 1:1 mixtures of human plasminogen and streptokinase are incubated in the presence of excess GdnBzoNph, again 0.7-0.8 moles of p-nitrophenolate are released per mole of plasminogen initially added. If excess [^{14}C]-AmSMeBzoNph is now added, no further p-nitrophenolate is released and no radioactivity is found in the complex. Thus, GdnBzoNph and AmSMeBzoNph compete for the same site in the complex. All these experiments are described in Table I.

Discussion - Previous studies (10) have demonstrated that AmSMeBzoNph was a reagent that would very rapidly acylate the active center serine residue of plasmin with concomitant release of p-nitrophenol. Since the reaction is instantaneous and very slow turnover of the acyl enzyme is noted, we felt that this reagent could be very useful in studying very early events in the plasminogen activation mechanism. In this case the activation reactions must be immediately terminated or proteolysis will occur in the activation mixtures. We chose the reagent [^{14}C]-AmSMeBzoNph to terminate these reactions although similar reagents, such as p-nitrophenyl-p'-guanidino benzoate (GdnBzoNph) were available, since the former reagent could be easily prepared radioactively in a relatively inexpensive manner and this compound like, GdnBzoNph, gives an immediate "burst" of p-nitrophenol when mixed with plasmin. The resulting acyl-plasmin is a relatively stable species. In this study we have shown the covalent incorporation

Table I

Incorporation of [^{14}C]-AmSMeBzoNph into the Plasminogen Moiety of the Streptokinase-Human Plasminogen Complex

Conditions of incubation [a]	p-nitrophenolate released (moles/mole)	[^{14}C] incorporated (moles/mole)
1 SK + 10 [^{14}C]-AmSMeBzoNph + 1 HPg for 2 minutes	0.70	0.75
1 SK + 10 GdnBzoNph + 1 HPg for 2 minutes + 10 [^{14}C]-AmSMeBzoNph for 2 minutes	0.72[b]	0

[a]Abbreviations are: SK-streptokinase; HPg-human plasminogen.

[b]The p-nitrophenolate released is derived from the GdnBzoNph.

of this reagent into human plasminogen, with the concomitant release of p-nitrophenol, in the presence of stoichiometric levels of streptokinase. Neither human plasminogen nor streptokinase alone would covalently incorporate this reagent at the time periods used here. We do have evidence however that at longer times of incubation, plasminogen or streptokinase alone will slowly covalently incorporate the reagent, probably non-specifically through nucleophilic attack by lysine residues. However this problem does not present itself in very short term experiments.

The studies described here give direct evidence for the

generation of an active site in human plasminogen accompanying its binding to streptokinase. This active site also binds the well known acylating agent, GdnBzoNph, and may well be the same active site which ultimately appears in human plasmin.

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