Interaction of Streptokinase and Human Plasminogen

IV. FURTHER GEL ELECTROPHORETIC STUDIES ON THE COMBINATION OF STREPTOKINASE WITH HUMAN PLASMINOGEN OR HUMAN PLASMIN*

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SUMMARY

Streptokinase is shown to react with either human plasmin or human plasminogen to form an equimolar product which migrates in starch gel electrophoresis at pH 6.0 or 8.0 with a migration rate intermediate between the rates of migration of the two proteins. When streptokinase was present in molar excess in the reaction mixtures, the uncombined streptokinase displayed a migration rate at pH 6.0 (but not at pH 8.0) which was different from that of native streptokinase. Inhibition of the enzymatic activity of the reaction product with diisopropyl phosphorofluoridate did not cause dissociation of the complex, although no reaction product was observed in starch gel electrophoresis with mixtures of streptokinase and diisopropylphosphoryl plasmin.

When equimolar mixtures of human plasminogen or human plasmin and streptokinase were subjected to electrophoresis at pH 6.0 in starch gels containing 6.4 M urea, the equimolar product was not observed; instead, plasmin and modified streptokinase were in evidence. On the other hand, when electrophoresis was carried out at pH 6.0 to 8.5 in urea-starch gels, reaction mixtures of human plasmin or human plasminogen and streptokinase displayed a number of electrophoretically distinct products which were derived from streptokinase. This marked degradation was not observed with human plasminogen-streptokinase reaction mixtures when the proteins were dissolved in 6.4 M urea prior to interaction and incubation, but it was noted in reaction mixtures made by mixing solutions of human plasmin and streptokinase which were individually dissolved in 6.4 M urea.

The equimolar reaction product formed by reaction of streptokinase with human plasminogen was subjected to gel filtration chromatography in lysine-tris(hydroxymethyl)aminomethane buffer, and the isolated main peak was shown to display potent bovine plasminogen activator activity and weak proteolytic activity.

The sequence of events occurring in the interaction of streptokinase with human plasminogen or human plasmin is discussed on the basis of the present findings and those reported previously from this laboratory. The results are taken to support the theory that the observed reaction product is a complex of human plasmin and structurally modified streptokinase.

Previous reports from this laboratory have described experiments on the interaction of highly purified human plasminogen and highly purified streptokinase in the presence of 6-aminocaproic acid. A main reaction product derived from an equimolar interaction was identified in both ultracentrifugal and gel electrophoresis experiments (1, 2). The molecular weight of the product was found to equal the sum of the weights of the two proteins, and its rate of electrophoretic migration was shown to be intermediate between the rates of migration of the two proteins. In the ultracentrifugation studies an excess of either protein was quantitatively identified as a boundary separate from the reaction product. Similarly, in the starch gel electrophoresis experiments at pH 8 to 8.5, an excess of either component was identified on the basis of its migration rate.

It was of interest to extend the electrophoretic studies on human plasminogen-streptokinase mixtures in order to establish more firmly the specificity of the observed reaction and to examine the products of reaction mixtures of human plasmin and streptokinase. The latter was particularly desirable since both human plasminogen and human plasmin are known to generate a potent plasminogen-activating activity upon interaction with streptokinase (3-5). Additionally, it seemed possible that further insight into the nature of the interaction might be gained by subjecting reaction mixtures to electrophoresis under dissociating conditions.

In the present studies, reaction mixtures of the interacting proteins at known molar ratios were incubated under various experimental conditions and subjected to electrophoresis in gels in the presence or absence of urea. Additionally, molecular sieve chromatography has been used to study the nature of the reaction product formed by the interaction of streptokinase and human plasminogen.
EXPERIMENTAL PROCEDURE

Materials

The preparation and partial characterization of highly purified streptokinase, human plasminogen, and plasmin prepared from the latter have been described previously (1, 2, 5, 6). The preparations of streptokinase displayed a specific activity of at least 80,000 (and in most cases greater than 90,000) streptokinase units per mg of protein and were chromatographed at least once on DEAE-cellulose (6). Minor impurities could be seen in electrophoretograms of some of these preparations. Human γ-globulin and human serum albumin have also been described (1). Crude bovine plasminogen used was the “working bovine plasmin” described by Hummel et al. (7). All other compounds have also been described previously (1, 2, 5, 6, 8). DFP was stored as a 0.5 M solution in 2-propanol (Merck). Sephadex G-200 (particle size, 40 to 120 μ) was purchased from Pharmacia.

Methods

Reaction Mixtures—Reaction mixtures of streptokinase and human plasminogen or plasmin of definite molar ratios were prepared as described previously (2, 5) and were incubated under various conditions prior to electrophoresis. Specific conditions for each experiment will be given under “Results.” Conditions with mixtures of other proteins will also be described in that section.

Starch Gel Electrophoresis—The methods described previously (2, 8) were used.

Gel Filtration Chromatography—Solutions of streptokinase, human plasminogen, and an approximately equimolar mixture of the two proteins were prepared in 0.1 M lysine-0.025 M Tris buffer, pH 8.0 (total volume, 3.5 ml in each case), and passed through a column (1.6 × 43 cm) of Sephadex G-200 which was equilibrated with the same buffer. The human plasminogen-streptokinase mixture was incubated at 30°C for 1 min and then cooled rapidly by immersion in an ice bath before application to the column. Elution was performed with the same lysine-Tris buffer at a flow rate of 6 to 10 ml per hour at 4°C. Fractions were collected with the aid of a Technicon fraction collector. The protein content of the samples collected was determined by measuring the absorbance at 280 μm. One absorbance unit of protein is defined as the concentration that shows an absorbance of 1.0 through a 1 cm light path.

Preparation of DIP-plasmin and DIP-activator—DIP-plasmin was made by incubating a plasmin solution (A280 approximately 10) in 0.1 M lysine-0.05 M Tris buffer at pH 8.0 in the presence of 0.015 M DFP (final 2-propanol concentration, approximately 3%, v/v) for 1 hour at 20°C. The DIP-plasmin was dialyzed exhaustively against 0.001 M HCl and precipitated by the addition of solid NaCl to give a 2 M NaCl solution. The precipitate was finally redissolved in water and dialyzed exhaustively against 0.001 M HCl to remove the NaCl. By proteolytic assay, greater than 99% of the activity was lost by this procedure. A control sample without DFP was treated in the same way. This latter sample lost about 30% of its proteolytic activity, presumably by autolysis, under the conditions of the experiment. DIP-activator was made essentially as described above except that an equimolar solution of streptokinase and human plasminogen in lysine-Tris buffer (A280 approximately 6) was incubated for 5 min prior to the addition of DFP. After incubation with DFP for 1 hour at 20°C, the solution was subjected to gel electrophoresis. A control mixture subjected to the same conditions except that DFP was omitted retained greater than 95% of its initial activator activity at the conclusion of the 1-hour incubation time. After removal of excess DFP by exhaustive dialysis against lysine-Tris buffer, pH 8.0, at 4°C, the treated sample retained less than 1% of its activator activity, whereas the control (i.e. the reaction mixture without DFP) retained about 70% of its activity.

Preparation of Alkali-denatured Streptokinase—A solution of streptokinase (A280 approximately 23) in 0.2 M NaOH was incubated at 35°C for 2 hours to abolish activity completely. The pH of the solution was adjusted to 7.5 with 1 N HCl. The solution was kept in an ice bath and used on the same day it was prepared.

Preparation of Autolyzed Human Plasmin—A solution of plasmin (A280 approximately 7) in lysine-Tris buffer, pH 7.5 to 8.0, was incubated at 37°C for 2 hours. Inactivation was about 95% complete at the end of this time. The solution was kept in an ice bath and used on the same day it was prepared.

Enzymatic Assays—Activator and proteolytic activities were determined by the procedures of Hummel et al. (7). In the activator assay the addition of excess streptokinase to the sample prior to the incubation with bovine plasminogen was, of course, not performed.

RESULTS

Starch Gel Electrophoresis of Reaction Mixtures of Streptokinase and Human Plasminogen or Human Plasmin—Fig. 1 shows electrophoretograms observed with reaction mixtures of either streptokinase and human plasmin (a, c, and e) or mixtures of streptokinase and human plasminogen (f, g, and i). In these experiments, reaction mixtures of the interacting proteins were subjected to electrophoresis in starch gel containing 0.2 M 6-aminocaproic acid, pH 8.5. Appropriate controls were also examined in the same experiment. The results with streptokinase-plasminogen mixtures confirm those reported previously (2). Moreover, it is evident that the interaction of human plasmin with streptokinase also results in the formation of a new zone with a migration rate intermediate between those of the two proteins. The product of human plasmin-streptokinase interaction appears to be a diffuse double zone, whereas the human plasminogen-streptokinase product appears to be less polydisperse. In fact, double zones similar to those shown for the plasmin-streptokinase product have been observed with plasminogen-streptokinase mixtures also (e.g. Fig. 1 of Reference 2), and single diffuse zones have been observed with plasmin-streptokinase mixtures. It is also apparent from the data of Fig. 1 that the migration rate of excess streptokinase is indistinguishable from that of unreacted streptokinase, although some degradation of the excess protein may be observed.

Fig. 2 shows the results of an experiment in which various plasminogen-streptokinase mixtures were incubated and subjected to electrophoresis at pH 6.0. In confirmation of previous results (2), the reaction product appears considerably more homogeneous under these conditions. Further, in contrast to the results shown in Fig. 1, when present in excess the streptokinase displayed a significantly different migration rate from that of unreacted

1 The abbreviations used are: DFP, diisopropyl phosphorofluoridate; DIP-, diisopropylphosphoryl-.
streptokinase, suggesting that the structure of the excess streptokinase was altered. When streptokinase was present in several fold molar excess, a zone corresponding to unreacted streptokinase was also in evidence (Fig. 2, g and i). In separate experiments it has been shown that prolongation of the incubation time (especially at pH 8 to 8.5 and at 37°C) gradually leads to the conversion of all of the excess streptokinase to the modified streptokinase species. On the other hand, the reason for the lack of appearance of excess plasminogen is uncertain (Fig. 2a), and further experimentation is required for clarification of this point.

Fig. 3 shows the results of experiments with human plasmin-streptokinase mixtures at pH 6.0. As with plasminogen-streptokinase mixtures at this pH, a more homogeneous reaction product was observed, and, when present in excess, streptokinase was modified.

**Specificity of Reaction Observed between Streptokinase and Human Plasminogen or Human Plasmin**—That the reaction product described in the first three figures is a specific product derived from the interaction of streptokinase and human plasminogen or human plasmin is supported by the following observations from experiments which were carried out under essentially the same conditions as described above (data are not presented in the interest of economy of space and because of their negative character). (a) Alkaline-denatured streptokinase, which shows a diffuse electrophoretic pattern, did not yield a product with either human plasmin or human plasminogen. (b) A reaction product was not seen in reaction mixtures of streptokinase and human γ-globulin or human serum albumin. Further, in an experiment in which albumin was added to an equimolar mixture of streptokinase and human plasminogen, the reaction product and albumin migrated separately with their characteristic migration rates.

(c) Reaction mixtures of autolysed human plasmin or bovine plasminogen^2 with streptokinase failed to show the presence of reaction product or of modified streptokinase.

In the experiment with bovine plasminogen it was not possible to prepare mixture with known molarities, since the bovine plas-
Fig. 4. Electrophoretograms of streptokinase-human DIP-plasmin mixtures and DIP-activator. In A, DIP-plasmin (see ‘Experimental Procedure’) was mixed with streptokinase at the designated molar ratios and incubated for 10 min at 20° prior to insertion into the gel. Controls were treated in the same way. a, DIP-plasmin; b, streptokinase-DIP-plasmin mixture, molar ratio of 0.8:1; c, streptokinase; d, streptokinase-DIP-plasmin mixture, molar ratio of 0.4:1. In B, the patterns of DIP-activator and of an untreated equimolar mixture of streptokinase and human plasminogen are shown. a, plasminogen; b, control mixture of streptokinase and human plasminogen, molar ratio of 1:1, incubated for 1 hour at 20°; c, streptokinase-human plasminogen mixture incubated for 1 hour at 20° in the presence of DFP (DIP-activator; see ‘Experimental Procedure’); d, streptokinase. In both A and B the buffer composition of the gel, reaction mixtures, and electrode vessels was 0.2 M 6-aminocaproic acid, 0.025 M Tris, and 0.1 M NaCl, adjusted to pH 6.1 with glacial acetic acid. Electrophoresis was performed at 12 volts per cm for 48 hours.

In all of the experiments described above, the proteins displayed the same migration rate and pattern whether they were incubated alone or as mixtures prior to electrophoresis.

Effect of DFP on Interaction—Human plasmin has been reported to be inhibited by treatment with DFP (9), and it was of interest, therefore, to study the interaction of human DIP-plasmin with streptokinase. The activator activity present in an equimolar mixture of streptokinase and human plasminogen or human plasmin (5) is also inhibited by treatment with DFP, and gel electrophoretic examination of DFP-activator was also performed. Fig. 4A shows the electrophoretograms of DIP-plasmin and reaction mixtures of DIP-plasmin and streptokinase at pH 6.0. There was no evidence for the presence of a reaction product, and both proteins migrated with their expected rates.

*Fig. 4A (b and c) shows the electrophoretograms of a DFP-treated equimolar mixture of human plasminogen and streptokinase (DIP-activator; see ‘Experimental Procedure”) and a control untreated equimolar mixture. As may be seen, their patterns are essentially identical.*

Electrophoresis of Streptokinase-Human Plasminogen or Human Plasmin Reaction Mixtures in Urea Starch Gel—Fig. 5 shows the patterns observed with an equimolar mixture of streptokinase and human plasminogen incubated for various times prior to
observed when human plasminogen and streptokinase were individually dissolved in 6.4 M urea prior to mixing (see Fig. 6). In this regard, it should be noted that some time elapses (5 to 10 min) between the insertion of the sample and the start of electrophoresis. As the time of incubation is prolonged (Fig. 5, e through f), the number and concentration of these degradation products increases while the intensity of the streptokinase band decreases, indicating that the bulk of the degradation products are derived from streptokinase. In support of this conclusion it is pertinent to point out that when mixtures with excess streptokinase were incubated for some time (e.g., 10 min) before electrophoresis the resultant patterns were essentially the same as those shown in Fig. 5, e through f, with the exception that the concentration of degradation products was greater. Furthermore, the patterns shown in Fig. 5 were not similar to the modified streptokinase observed when reaction product is readily discernible, and that no reaction product was observed, and instead modified streptokinase was apparent along with a zone corresponding to human plasmin. Eluates of the plasmin zone displayed proteolytic activity when tested with appropriate substrates (2). (Results were erratic, and in some experiments the eluates were enzymatically inactive.)

The modified streptokinase evident in Fig. 7 appears to be similar to the modified streptokinase observed when reaction mixtures containing excess streptokinase were subjected to electrophoresis at pH 6.0 in the absence of urea (Figs. 3 and 6). Even in the mixture which was mixed and immediately inserted into the well (Fig. 7a), a small amount of modified streptokinase was in evidence. On the other hand, as at pH 8.5, if both streptokinase and human plasminogen were individually dissolved in 6.4 M urea prior to mixing and then subjected to electrophoresis at pH 6.0 in urea gel, essentially unaltered streptokinase and plasminogen were observed (data not shown).

**Fig. 7.** Electrophoretograms of human plasminogen-streptokinase mixtures in urea-starch gel at pH 6.0. All mixtures were prepared in 0.2 M 6-aminocaproic acid, 0.055 M Tris, and 0.1 M NaCl, pH 6.0, at a plasminogen to streptokinase molar ratio of 1:1.3. The samples were incubated for the designated times and at the designated temperatures prior to insertion into the gel. Buffer composition of starch gel was 0.2 M 6-aminocaproic acid-6.4 M urea at pH 6.0. Buffer composition of electrode vessels was 0.2 M 6-aminocaproic acid adjusted to pH 6.0 with glacial acetic acid. Electrophoresis was performed at 12 volts per cm for 4 hours...
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Aggregation of the equimolar product is apparently influenced by 
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Fig. 8. Elution patterns of streptokinase (A), human plasminogen (B), and an equimolar mixture (C) passed through a column of Sephadex G-200. ---, absorbance at 280 nm; --- (C), bovine plasminogen activator activity (7). The following amounts (in absorbance units; see "Experimental Procedure") were applied to the column: A, 7; B, 11; C, 14.4

of this solution at pH 6.0 (under the conditions shown in Fig. 2 or 3) revealed a single zone with a migration rate indistinguishable from the main reaction product observed in an equimolar mixture of streptokinase and human plasminogen or human plasmin.

Discussion

In previous reports from this laboratory (1, 2), the results of ultracentrifugal and gel electrophoretic experiments provided evidence that streptokinase and human plasminogen form an equimolar complex in buffers containing the potent inhibitor of plasminogen activation, 6-aminocaproic acid. The results shown in Fig. 8 of the present paper provide additional direct evidence for the combination of these two proteins. As may be seen in Fig. 8, a mixture of the two proteins in lysine-Tris buffer at pH 8.0 was clearly not retarded to the same degree as the individual proteins on passage through a Sephadex G-200 column, and both the protein and the activator activity emerged as a single main peak.

The fact that such combination occurred in lysine buffers indicates further, in confirmation of results obtained from enzymatic analyses of reaction mixtures (5), that the equimolar interaction is not dependent upon the presence of 6-aminocaproic acid. Ultracentrifugal analyses revealed higher molecular weight products to be present in equimolar reaction mixtures dissolved in lysine buffers at pH 7.5 as compared to 6-aminocaproic acid buffers at the same pH (1). Hence, the final state of aggregation of the equimolar product is apparently influenced by the presence of one or the other of the amino acids.

The present experiments (Figs. 1 and 3) extend previous findings to show that human plasmin also forms an equimolar reaction product with streptokinase. By the method used, this product was indistinguishable from that observed with streptokinase-human plasminogen mixtures. Confirmation of our previous conclusion (1, 2, 5) that the product is the result of a specific interaction between streptokinase and human plasminogen or human plasmin was provided by the facts that the reaction product was not observed with mixtures of denatured streptokinase and human plasminogen or human plasmin, or with mixtures of native streptokinase and bovine plasminogen, human serum albumin, human γ-globulin, autolyzed human plasmin, or DIP-plasmin.

In an attempt to diminish the likelihood of producing artifacts arising from nonspecific proteolytic cleavage at slightly alkaline pH, several of the experiments presently reported were carried out at pH 6.0. Such experiments revealed that with mixtures of either human plasminogen or plasmin and an excess of streptokinase the reaction product was observed and, in addition, at least a part of the excess streptokinase displayed a migration rate which was different from native unreacted streptokinase (Fig. 2). Such a finding was not evident either in the present studies (Fig. 1) or in those reported previously (2) when electrophoresis was carried out at pH 8 to 8.5. The present observations are thus interpreted to indicate that, when present in excess, streptokinase is modified in structure in such a manner that it is electrophoretically distinguishable from native streptokinase at pH 6.0 but not at pH 8.0.

In an attempt to define more clearly the nature of the observed product, reaction mixtures were subjected to electrophoresis in urea-gel. Under these conditions no significant concentration of reaction product was observed either at pH 6.0 or at pH 8 to 8.5. However, at either pH, alteration of streptokinase was observed when the proteins were incubated in the absence of urea prior to electrophoresis. At pH 6.0, zones corresponding to a modified streptokinase4 and plasmin were observed upon electrophoresis of a human plasminogen-streptokinase mixture, while at pH 8 to 8.5, extensive degradation of streptokinase was observed with mixtures of either human plasminogen or plasmin and streptokinase.

On the other hand, the electrophoretic patterns more closely resembled those of a mixture of the unreacted proteins when both streptokinase and human plasminogen were individually dissolved in 6.4 M urea prior to mixing (Fig. 6, g and h), indicating that significant interaction did not take place in the presence of high concentrations of urea. It seems reasonable to account for this fact on the basis of alteration of the configurations of one or both of the proteins in 6.4 M urea in such a way that formation of the product is not permitted. Nevertheless, the presence of urea prior to mixing did not prevent the degradation of streptokinase by human plasmin (Fig. 6, c and d), although formation of the stable activator complex was largely prevented. The results indicate, then, that modification of streptokinase is observed in urea-gels only when plasminogen and streptokinase are mixed in the absence of urea, i.e. only after the generation of the proteolytic center in the reaction product.

4 There is no intention to imply that the modified streptokinase observed under these conditions is identical with the modified streptokinase observed in reaction mixtures containing streptokinase in excess and subjected to electrophoresis at pH 6.0 in the absence of ureas. This may be so, but the present data neither prove nor disprove this point.
When these findings are considered together with those reported previously (1, 2, 5), the following reaction scheme may be presented. In this representation the following abbreviations are used: HPg, human plasminogen; "HPg" conformationally altered human plasminogen; HP, human plasmin; SK, streptokinase; SK', modified streptokinase.

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\text{HPg} + \text{SK} \rightarrow \text{["HPg"-SK]} \\
\text{HP} + \text{SK} \rightarrow \text{[HP-SK] } \rightarrow \text{HP-SK'}
\]

In the simpler case, that of the interaction of plasmin and streptokinase, it has been shown that plasmin activated by DFP does not interact with streptokinase to form a complex stable in an electrical field. On the other hand, inactivation of the activator with DFP does not dissociate the product (Fig. 4). Thus, it would seem that the initial site of attachment is at or near the active center. Thereafter, conformational changes probably occur in either or both proteins to permit the formation of a more stable product involving several sites of complexation. This interpretation assumes that the DFP-sensitive centers in free plasmin and in plasmin to which streptokinase is attached (i.e. the main reaction product observed in the electrophoresograms, the activator enzyme) are identical, an assumption which is supported by preliminary experiments in our laboratory with radioactively labeled DFP, which indicate that the radioactively labeled peptides liberated by tryptic digestion of DFP-plasmin and DFP-activator are identical.

In the case of the interaction of human plasminogen and streptokinase, the simplest mechanism would invoke conformational changes in the plasminogen molecule upon interaction with streptokinase, which result in the formation of a complex of transient existence with the active center exposed. Conversion to the plasmin-streptokinase complex then occurs, perhaps by cleavage of a peptide bond, and the ultimate plasmin-modified streptokinase complex, which probably represents the final enzymatically active stable form of activator, is formed by cleavage of additional peptide bonds. It should be remembered that all of these reactions occur rapidly and that under appropriate conditions the plasmin-modified streptokinase complex may form within minutes at neutral pH (5).

Whether or not the modification of streptokinase is an obligatory outcome of the interaction of streptokinase with human plasminogen seems an important point to resolve since, if this were so, other mechanistic interpretations could be advanced. However, the simplest explanation for the demonstration of the presence of modified streptokinase in reaction mixtures is that it arises as a result of autodigestion after the formation of the enzymatic center in the complex. It is relevant that in experiments with impure preparations of streptokinase and human plasminogen the activator activity has long been known to be unstable at neutrality, more so at pH 7.5 than at pH 8.0 (10), and to be protected by the presence of excess streptokinase (11). Furthermore, weak (relative to human plasmin) proteolytic activity with casein has been reported in activator preparations by several investigators (e.g. Reference 5). These findings clearly suggest that the activator complex is capable of self-digestion. The data presented in this paper would appear to have general application in serving to demonstrate the problems inherent in attempting to distinguish between true reaction products and artifactual products in a system which involves a proteolytic enzyme both as an interacting component and as a product of the reaction.

A final point concerning the general conclusions of this paper appears to be in order. For some time a streptokinase-human plasmin complex has been postulated by many investigators to be responsible for high activator activity (3, 4, 12-16). Indeed, in 1954, Sherry (12), working with reaction mixtures of impure proteins, made such a postulate on the basis of clot lysis studies in vitro. Furthermore, on the basis of acid inactivation studies, Blatt et al. (13, 14) suggested that a human plasmin-modified streptokinase complex was the species responsible for high activator activity. The findings of the present paper, along with those reported previously (1, 2), may well provide direct physicochemical evidence for the existence of such products in reaction mixtures of streptokinase and human plasminogen or human plasmin.

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