Interaction of Streptokinase and Human Plasminogen

1. COMBINING OF STREPTOKINASE AND PLASMINOGEN OBSERVED IN THE ULTRACENTRIFUGE UNDER A VARIETY OF EXPERIMENTAL CONDITIONS

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The activation of human plasminogen by streptokinase has been the subject of extensive investigation (4-8). There appears to be general agreement that at least two enzymatic activities are demonstrable in streptokinase-plasminogen reaction mixtures. One is the proteolytic enzyme human plasmin, characterized in part by its ability to hydrolyze lysine and arginine esters and protein substrates such as casein and fibrin. The second enzyme, also active with basic amino acid esters, is chiefly distinguished by the property of enzymatically converting to plasmin a species of plasminogen which is refractory to activation by streptokinase alone. Bovine plasminogen is an example of such a species and has been used extensively as a substrate for detecting the "activator" activity in a streptokinase-human plasminogen reaction mixture. Bovine plasminogen is not activated by streptokinase alone, by human plasminogen alone, or by human plasmin alone. In their studies on bovine plasminogen activation, Mändert and Lassen (4, 5) postulated that streptokinase interacted with a substance, "proactivator," invariably present in human plasminogen preparations, to produce an activator of bovine plasminogen. The same theory has been advanced by Trelle and Sherry (9) for the activation of human plasminogen; i.e. a non-enzymatic reaction of streptokinase with "proactivator" has been postulated to precede the enzymatic conversion of human plasminogen to plasmin. The existence of "proactivator" distinct from human plasminogen remains largely hypothetical, and, in fact, evidence for their possible identity has been reported (10, 11).

Little definitive work has been done to elucidate the chemical sequence of events in the interaction of streptokinase and human plasminogen. Virtually nothing is known about the molecular nature of the products formed in the interaction. Undoubtedly the chief reason for this is the fact that several reactions, viz. the generation of activator, activation of plasminogen, and auto-digestion of the enzymes, are occurring simultaneously. Nevertheless, kinetic studies in several laboratories have indicated that the relative concentrations of human plasmin and bovine plasminogen activator demonstrable in a streptokinase-human plasminogen reaction mixture depend primarily upon the ratio of streptokinase and plasminogen. When streptokinase is present in high concentration relative to plasminogen, activator activity predominates, whereas an excess of human plasminogen favors the generation of plasmin activity (5-7, 12, 13).

The present studies were undertaken to study the interaction of streptokinase and human plasminogen in an attempt to characterize physicochemically the enzymes formed in their interaction. With the use of highly purified plasminogen and streptokinase at definite molar ratios in systems containing inhibitors of "activator" and plasmin, it was possible to characterize partially a product of their reaction in ultracentrifugal and gel electrophoretic studies. The present paper describes the results of the ultracentrifugal experiments. Data on the physical characterization of purified streptokinase, a prerequisite for these studies, are also included.

EXPERIMENTAL PROCEDURE

Materials

Buffer Components—Inorganic compounds were Merck or Fisher "reagent grade" chemicals. DFP and e-aminocaproic acid were purchased from the Aldrich Chemical Company. The purity of the acid was satisfactory as judged by elemental, infrared, and paper chromatographic analyses. L-Lysine monohydrochloride was a high grade preparation (Nutritional Biochemicals).

Proteins—Streptokinase was purified by ion exchange chromatography,2 dialyzed against distilled water at 5° for about 24 hours to remove most of the salts, and lyophilized. It showed a high degree of homogeneity both by ultracentrifugation (see below) and in starch gel electrophoresis (3).

Highly purified human plasminogen was prepared from dried plasma Fraction III3 as described by Hagan, Ablondi, and De Renzo (10) or by extraction of a partially purified preparation with lysine as described by Hagan (14). The latter procedure has been further elaborated by Kline and Fishman (15). The preparations were dialyzed against 0.001 M HCl and kept either in the frozen state or at 4° until used. As will be evident below, the plasminogen preparations used in this work had a high order of purity as judged by ultracentrifugal analyses. Commonly about 5 to 10% of somewhat slower sedimenting material could be resolved. In contrast, by starch gel electrophoresis at neutral or alkaline pH, the plasminogen samples showed considerable polydispersity (3).

γ-Globulin was a commercial product of human origin, "im-

1 The abbreviation used is: DFP, diisopropyl phosphofluoridate.
2 Unpublished experiments.
3 Fraction III was obtained through the courtesy of Dr. T. Gerlough of E. R. Squibb and Sons and Dr. J. N. Ashworth of the American Red Cross.
r~e globulin" (Lederle). Its purity was established by electrophoresis and ultracentrifugation.

Bovine serum albumin was a crystalline product supplied by Armour.

Methods

Velocity Sedimentation Analyses—A Spinco model E ultracentrifuge equipped with a phase plate and an RTIC unit was used. Samples were always equilibrated with the appropriate buffer by dialysis, after which the sample and dialysate were usually centrifuged simultaneously in a double sector cell. Sedimentation coefficients were calculated from enlarged images of the schlieren patterns obtained from velocity centrifugation experiments. When used for the characterization of streptokinase, they were converted in the usual way to their values in water at 20° and extrapolated to infinite dilution. In interaction experiments, no attempt was made to correct sedimentation coefficients for variability of the dialysate viscosity or for the protein concentration. However, a correction was made for the variation of viscosity with temperature by using values reported for water.

For homogeneity estimations, the schlieren patterns were graphically corrected for the curvature of the base-line. However, a correction was made for the variation of viscosity with temperature by using values reported for water.

Analyses were carried out on the streptokinase dissolved in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.1 M NaCl, pH 3.0. The molarities of the plasminogen and streptokinase solutions were calculated from their molecular weights and the areas under their schlieren patterns in these preliminary analyses. Sufficient streptokinase was then dissolved in 0.1 M sodium phosphate buffer, pH 7.4 to 7.5, containing 0.1 M e-aminocaproic acid, so that it could be conveniently mixed with the plasminogen solution to give the desired molar ratio and a total protein concentration of about 0.5%. Solid e-aminocaproic acid was added to the plasminogen and streptokinase solutions. When DFP was used it was added at a concentration of 0.01 M to both the plasminogen and streptokinase solutions and to the equilibrating buffer. The various buffer systems did not significantly influence the homogeneity or $s$ values of the individual proteins.

The two protein solutions were finally combined in volumes which gave the desired molar ratios, and the reaction mixture was subjected immediately to ultracentrifugation. Streptokinase-plasminogen mixtures were centrifuged at low temperature to minimize possible enzymatic activity. Some experiments with excess plasminogen were done at room temperature to facilitate resolution of the boundaries.

In control experiments with albumin or $\gamma$-globulin, the equilibration procedure was the same as that described above.

RESULTS

Physical Properties of Plasminogen and Streptokinase

The physical properties of plasminogen preparations of the type employed in this work have been previously reported (17). The molecular weight of 84,000 calculated from sedimentation and diffusion coefficients was checked by the short column sedimentation equilibrium method, which gave a value of 87,000. Results obtained in our laboratory by the equilibrium method with known proteins ranging from 13,000 to 160,000 in molecular weight indicated that under our conditions an error of 5 to 8% could be expected. Therefore, this value of 87,000 for plasminogen is considered to be a good confirmation of the value obtained by sedimentation and diffusion.

The physical properties of streptokinase were determined in phosphate buffer, pH 7.5. The schlieren centrifuge pattern for a streptokinase preparation typical of those used in these studies is shown in Fig. 1. It can be seen that the preparations had a high order of purity. There was no apparent dependence of $s$ on concentration, the value of $s_{0.02}$ being 3.2 ± 0.1 S from 0.1 to 0.5% concentration. Table I shows values of the diffusion constant obtained for 0.3% solution of streptokinase. The average value, after correction to water at 20°, was $6.3 \times 10^{-4} \text{ cm}^2 \text{g}^{-1} \text{s}^{-1}$.

The partial specific volume of one batch of streptokinase was found to be 0.745 cm$^3$ g$^{-1}$. In the calculations reported here, 0.75 was used as the value of this parameter. Insertion of the values for $s$, $D$, and $\Theta$ in the Svedberg equation yielded a molecular weight of 47,000. The intrinsic viscosity was found to be 0.07 cm$^3$ g$^{-1}$, and the viscosity increment (100[η]/η), about 9. On the assumption that the molecule is a rigid, unhydrated prolate ellipsoid, a molecular axial ratio (18) and frictional ratio (19) of about 7 and 1.4, respectively, were derived from these values.

Interaction of Streptokinase and Plasminogen in Presence of $\varepsilon$-Aminocaproic Acid

In preliminary experiments (1, 2), interaction of streptokinase and plasminogen was studied in the presence of both $\varepsilon$-aminocaproic acid and DFP. DFP was included in the reaction mixture since it has been reported to be an inhibitor of plasmin (20) and it seemed desirable to eliminate potential proteolytic degradation that might result from the presence of a small amount of plasmin contaminant in the plasminogen. The results obtained...
tained in those experiments will not be presented in detail here, since essentially the same results were observed in the presence or absence of DFP.

Fig. 2, a to e, shows schlieren patterns of streptokinase, plasminogen, and mixtures in 1:1, 2:1, and 1:2 molar ratios as indicated. Sedimentation coefficients for streptokinase and plasminogen separately had average values of 2.9 and 4.1 S, respectively, at approximately 0.5% concentration. These values were uncorrected for buffer viscosity or concentration dependence. When the two proteins were allowed to react at a 1:1 molar ratio, a single boundary appeared with a sedimentation coefficient which averaged 5.2 S over all the experiments (Fig. 2c). When excess streptokinase was present in the reaction mixture, the sedimentation pattern showed two boundaries (Fig. 2d). The pattern could be resolved into a 2.9 S component and a 5.2 S component. The area under the 2.9 S component was found to correspond closely to the mole excess of streptokinase. With plasminogen in excess, the sedimentation rates of the reaction product and the excess plasminogen did not give as clear a separation, but the skewed pattern could be resolved into a 4.1 S component and 5.2 S component (Fig. 2e). The amount of 4.1 S component corresponded closely to the mole excess of plasminogen. All of the values given above represent averages for a number of experiments. In any particular experiment, the values of the sedimentation coefficients were within 0.2 of the average. In most of the experiments, about 10% of the total protein sedimented more slowly than the reactants. This can probably be attributed to an impurity in the reactants or to a by-product.

### Table I

<table>
<thead>
<tr>
<th>Diffusion coefficient of streptokinase</th>
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<tr>
<td><strong>Diffusion coefficient corrected to water at 20° = 6.5 × 10⁻⁷ cm² g⁻¹</strong></td>
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<tr>
<td><strong>Time</strong></td>
<td><strong>Observed diffusion coefficient</strong></td>
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</table>

**Fig. 1.** Ultracentrifugal pattern of streptokinase used in physical characterization studies; 48-minute picture; 52,640 r.p.m.; phosphate buffer, [gamma] = 0.15, pH 7.5; 23.8°.

**Fig. 2.** Ultracentrifugal patterns obtained in experiments on the interaction of streptokinase and plasminogen in 0.1 M phosphate-0.1 M e-aminocaproic acid, pH 7.4, at 52,640 r.p.m. in double sector cells. a, streptokinase; 64-minute picture; 8.0°; single 2.9 S component. b, plasminogen; 72-minute picture; 19.6°; single 4.1 S component. c, streptokinase and plasminogen mixed at a 1:1 molar ratio; 72-minute picture; 8.7°; single 5.2 S component. d, streptokinase and plasminogen mixed at a 2:1 molar ratio; 72-minute picture; 13.0°; a 2.9 S and a 5.2 S component are present. e, streptokinase and plasminogen mixed at a 1:2 molar ratio; 88-minute picture; 18.9°; skewed peak can be resolved into a 4.1 S and a 5.2 S component.

**Fig. 3.** Ultracentrifugal patterns obtained by sedimenting a mixture of human γ-globulin and either streptokinase or plasminogen at 52,640 r.p.m. in double sector cells. a, streptokinase and γ-globulin in 0.1 M phosphate-0.1 M e-aminocaproic acid, pH 7.5, at 21.9°; 32-minute picture; 2.9 S and 7 S components are clearly separated. b, plasminogen and γ-globulin in 0.1 M phosphate-0.1 M e-aminocaproic acid, pH 7.4, at 13.3°; 72-minute picture; 4.1 S and 7 S components are clearly separated.

**Fig. 4.** Ultracentrifugal patterns obtained in experiments on the interaction of streptokinase and plasminogen in 0.1 M phosphate-0.1 M lysine, pH 7.3, at 52,640 r.p.m. in a synthetic boundary cell. a, streptokinase and plasminogen in a 1:1 molar ratio; 40-minute picture; 8.8°. The fast moving component is 8 to 10 S material. b, same system as a, but e-aminocaproic acid was added to a concentration of 0.1 M; 48-minute picture; 11.7°. Essentially a single 5.2 S component is present with a small amount of slower sedimenting material.
On the assumption of a mole for mole reaction, theoretical values of the relative areas of the schlieren peaks were calculated for the reaction mixtures of streptokinase and plasminogen of varying molar ratios. Table II shows these data along with the experimentally determined areas in one complete experiment with one batch of plasminogen.

As a check of the specificity of the reaction observed, it was desirable to analyze mixtures of streptokinase or plasminogen with other serum proteins. Each protein was therefore mixed with human γ-globulin of known concentration under conditions similar to those described above. Fig. 3, a and b, shows the schlieren patterns obtained from those experiments. In both cases the two proteins were resolved with appropriate sedimentation coefficients and concentrations. A similar experiment was done with streptokinase and serum albumin. Since their $s$ values are quite similar, the separation was not as clear-cut as with γ-globulin, but the boundary was obviously polydisperse and no component with $s$ in excess of that of albumin appeared.

To obtain the molecular weight of the 5.2 S reaction product, streptokinase and plasminogen were combined in a mole to mole ratio in 0.1 M phosphate buffer, pH 7.5, containing 0.1 M ε-aminocaproic acid, and immediately subjected to analysis by the Yphantis short column equilibrium sedimentation technique with schlieren optics (16). A weight average molecular weight of 117,000 was obtained. The plasminogen used in this particular experiment had 5 to 10% low molecular weight impurity. In the synthetic boundary cell, about 10% of the total protein in the reaction mixture appeared as very slowly sedimenting, polydisperse material, all of which was estimated to be of less than 5,000 molecular weight. The rest of the sample migrated as a single 5.1 S boundary. A molecular weight of 5,000 was arbitrarily assigned to all of the light material, and on this assumption the molecular weight of the 5.1 S component was calculated to be 130,000.

### Interaction of Streptokinase and Plasminogen in Presence of Lysine

Lysine is a much weaker inhibitor of plasminogen activation than ε-aminocaproic acid. It was of obvious interest, therefore, to study the interaction of streptokinase and plasminogen in the presence of lysine.

When 0.1 M lysine was used instead of the aminocaproic acid, both plasminogen and streptokinase individually had sedimentation rates and displayed homogeneity comparable to the results shown with aminocaproic acid. On the other hand, when the two proteins were combined at a 1:1 molar ratio in the presence of lysine, a new boundary with $s_{20}$ varying from 8 to 10 S appeared. This boundary represented about 60% of the total protein. In addition, polydisperse material with sedimentation coefficients ranging from 0 to 4 S were also observed. Fig. 4a illustrates this result. As in the previous experiments, essentially all of the protein was accounted for.

In another experiment, after streptokinase and plasminogen were combined at a molar ratio of 1:1 in lysine-phosphate buffer, the system was dialyzed against phosphate buffer containing 0.1 M ε-aminocaproic acid. When this procedure was followed, the pattern reverted to the type obtained with the aminocaproic acid alone. A main component, representing 90% of the total material, sedimented with $s_{20} = 5.4$ S, and the remainder moved more slowly. The same reversibility could be demonstrated merely by adding ε-aminocaproic acid at a concentration of 0.1 M to the system containing lysine and a 1:1 molar mixture of the two proteins. Fig. 4b illustrates this effect. By either means of addition of aminocaproic acid, the total protein was still accounted for in the schlieren pattern.

### DISCUSSION

Study of the interaction of streptokinase and human plasminogen in the ultracentrifuge involves two major difficulties. First, the plasminogen preparations are only slightly soluble at neutral pH values. Second, the activation of plasminogen by streptokinase proceeds so rapidly that the proteolytic enzyme plasmin is generated, hydrolyzes other proteins present, and digests itself. The result is a complicated mixture of products. It was hoped that the addition of ε-aminocaproic acid to reaction mixtures might eliminate these obstacles since it solubilizes plasminogen at neutrality in sufficient concentration to be easily observed in the ultracentrifuge and also inhibits the activation of plasminogen and the activity of plasmin (21). The results of our experiments indicate that this goal was realized.

In the presence of ε-aminocaproic acid, a complex with an $s_{20}$ of about 5.2 S was observed when streptokinase and plasminogen were allowed to react at equimolar concentrations. As previously pointed out, this $s_{20}$ value is uncorrected for the viscosity of the medium or for concentration dependence. When streptokinase was in excess, an additional boundary appeared. The $s_{20}$ value of this new boundary was very close to that of streptokinase and quantitatively corresponded to the molar excess of streptokinase. When plasminogen was in excess, a shoulder appeared which was not clearly resolved, probably because of the closeness in values of the sedimentation coefficients of plasminogen and the complex.

The nature of the schlieren patterns when ε-aminocaproic acid was used indicates that the complex formation is an irreversible process, or that, if an equilibrium exists, the formation of the complex is greatly favored. This finding is in accord with results obtained in a study of streptokinase-plasminogen interaction under similar conditions by starch gel electrophoresis (3).

As illustrated in Table II, at all molar ratios of streptokinase and plasminogen in the presence of the aminocaproic acid, the relative areas of the schlieren patterns corresponded very closely to theoretical values calculated on the assumption of a 1:1 molar combination. These results support the conclusion that a 1:1 molar combination did indeed occur. In addition, these results indicate that the molecular weight values for the individual pro-

### Table II

<table>
<thead>
<tr>
<th>Molar ratio of streptokinase to plasminogen</th>
<th>Relative areas and $s$ values of schlieren boundaries</th>
<th>Theoretical*</th>
<th>Observed</th>
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<tr>
<td></td>
<td>%</td>
<td>$s \times 10^4$</td>
<td>%</td>
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<tr>
<td>1:1</td>
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<tr>
<td></td>
<td>39</td>
<td>36</td>
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</table>

* Based on the assumption of a 1:1 molar interaction.
Lysine for aminocaproic acid was therefore considered to provide a system which more nearly approached natural conditions. In separate experiments it has been established that an equimolar mixture of streptokinase and plasminogen in 0.1 m phosphate-0.1 m lysine, pH 7.4, has barely detectable proteolytic activity but is capable of activating bovine plasminogen.

A complex centrifugal pattern was obtained when streptokinase and plasminogen were permitted to react at a 1:1 molar ratio in the presence of lysine. In addition to a fast sedimenting, 8 to 10 S boundary, the pattern revealed the presence of more slowly sedimenting, polydisperse material. The simple addition of e-aminocaproic acid was found to convert this pattern to the less complex pattern of essentially a single 5.4 S boundary, i.e., to the pattern observed in the presence of the aminocaproic acid alone. An association therefore apparently occurs in the presence of lysine which does not occur in the presence of e-aminocaproic acid and which is readily reversed by the presence of this acid. Clarification of this phenomenon would contribute considerably to an understanding of the streptokinase-plasminogen interaction. However, results obtained thus far both by ultracentrifugation and by other methods make it evident that such a study will be a complicated problem in itself. Therefore it is our intention to make it the subject of a separate report after conclusive data are obtained.

SUMMARY

1. The interaction of streptokinase and human plasminogen has been studied by ultracentrifugation at pH 7.5 in the presence of e-aminocaproic acid, which is a solubilizing agent for plasminogen at neutral pH values and an inhibitor of its conversion to plasmin.

2. Under such conditions, an equimolar mixture of highly purified streptokinase and plasminogen produced a single 5.2 S boundary. An excess of either reactant could be quantitatively identified as a boundary separate from this reaction product.

3. The molecular weight of the 5.2 S complex was determined by equilibrium centrifugation to be about 130,000, which is very close to the sum of the molecular weights of streptokinase (47,000) and plasminogen (84,000), but it is not possible to state whether a simple additive reaction took place.

4. In control experiments, no changes in sedimentation properties or homogeneity of streptokinase, plasminogen, albumin, or γ-globulin were observed in the presence of e-aminocaproic acid. Also, combination did not take place between streptokinase or plasminogen and albumin or γ-globulin.

5. When lysine was substituted for e-aminocaproic acid, a complex centrifugal pattern was obtained with an equimolar mixture of streptokinase and plasminogen, 60% of the total protein appearing as an 8 to 10 S component and 40% as polydisperse, lower molecular weight material. Dialysis of this system to substitute e-aminocaproic acid for lysine or addition of e-aminocaproic acid without removal of lysine restored the system to the pattern obtained with e-aminocaproic acid alone.

6. The implications of all the above results in the process of plasminogen activation by streptokinase are discussed.

7. Some of the physical properties of streptokinase are reported.

Acknowledgments—It is a pleasure to acknowledge the participation of Dr. R. A. Brown, presently at Oak Ridge, and Messrs. F. B. Ablondi and J. J. Hagan in the initial phases of these studies. We are also grateful to Dr. S. N. Timesheff, of the
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