THE ACTIVATION OF HUMAN PLASMINOGEN BY STREPTOKINASE*

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Human plasma contains a proteolytic enzyme precursor termed plasminogen (1) or profibrinolysin (2), which, upon activation by physical agents or specific bacterial kinases, is converted to plasmin (1) or fibrinolysin (2). Streptokinase (henceforth referred to as SK), an extracellular hemolytic streptococcal product, has been a commonly used activator for human plasminogen. Two mechanisms have been proposed for the activation of plasminogen by SK: (a) plasminogen is converted to plasmin, a new substance, by enzymatic activation (3) and (b) the conversion of plasminogen to plasmin results from a “stoichiometric” interaction of SK with plasminogen (4, 5).

Studies with synthetic substrates on the activation of plasminogen by SK reveal that human plasminogen preparations contain two factors: a proactivator, converted by SK in stoichiometric fashion to a plasminogen activator, and plasminogen, which is enzymatically converted by the activator to the proteolytic enzyme plasmin. Evidence for a two-step activation of plasminogen by SK, in agreement with that recently suggested by others (6, 7), was obtained through three types of studies: (a) fractionation experiments demonstrating two factors in SK-activated plasminogen preparations, i.e. a lysine esterase alone, and a proteolytic enzyme capable of splitting casein, arginine, and lysine esters; (b) inhibition experiments relating the lysine esterase to the plasminogen activator; and (c) activation experiments demonstrating the reversible stoichiometric activation of the lysine esterase by SK and the enzymatic activation of the proteolytic enzyme.

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Plasminogen preparations were prepared according to the method of Kline (8) from human plasma Fraction III. A commercial preparation of SK was used. Lysine ethyl ester (LEe), tosylarginine methyl ester (TAMe), and tosylarginine butyl ester (TABe) were used as substrates and prepared as described (9).

Fibrinolytic activity, with a bovine fibrinogen substrate, casein proteolysis, and TAMe splitting were measured by reported methods (9).

A photometric assay for TABe hydrolysis, which could be employed in the presence of LEe, was developed. The principle of the method consisted in removing the substrate, following enzymatic digestion, by precipitation with ammonium sulfate and determining the tosylarginine in solution. The enzymatic digestion was carried out in 20 per cent methyl Cellosolve because of the slight solubility of TABe in water. The procedure was as follows: 0.2 ml. of a 0.1 M solution of TABe in methyl Cellosolve was added to a digestion mixture containing 0.5 ml. of tris(hydroxymethyl)aminomethane (Tris) buffer (pH 9) and 0.3 ml. of enzyme solution in a 12 ml. glass centrifuge tube. At the end of digestion time, 4 ml. of cold saturated ammonium sulfate solution were added, the mixture was allowed to stand for 30 minutes, and the precipitated TABe removed by centrifugation. Blanks were prepared by reversing the addition of ammonium sulfate and TABe. 1 ml. aliquots of the supernatant fluids were diluted to 25 ml. with water and the optical density was determined at 230 μm in a Beckman spectrophotometer with photomultiplier attachment. The difference between the blank and test sample represents the tosylarginine liberated by enzymatic digestion. Tosylarginine has an ultraviolet absorption spectrum with a 230 μm maximum and a millimolar extinction of 12.05. The sensitivity of the method may be seen from a standard curve obtained with 0.1 to 0.8 μg of crystalline trypsin (Fig. 1).

The assay of LEe esterase was modified from the original method (9) by separating the lysine formed during digestion from the substrate. To 1 ml. of the digestion mixture, identical with the one described previously, 10 ml. of acetone were added and the mixture was allowed to stand for 30 minutes. The precipitated lysine, after separation by centrifugation and decantation of the supernatant fluid, was dissolved in 2 ml. of water. 2 ml. of formaldehyde (37 per cent neutralized to phenolphthalein pink)
were added, and the mixture was titrated with 0.05 N sodium hydroxide with a 1 ml. micro burette.

\[ \text{FIG. 1} \]

**Fig. 1.** Standard curve for trypsin assay by the photometric TADe method. 30 minutes digestion at 37°. The method is as described in the text.

**Fig. 2.** LEe and TAMe esterase activity of plasminogen preparation (15 γ of nitrogen per ml.) activated by several concentrations of SK (expressed as log, of SK units per ml.). LEe esterase and TAMe esterase activity was measured as described in the text and expressed as micromoles of ester split, per 30 minutes at 37° per ml.

**Results**

**Evidence for Two Factors in Plasminogen**

Previous studies had demonstrated that plasmin preparations attacked esters of the basic amino acids, arginine and lysine, but, upon purification of plasminogen and subsequent activation, the ratio of attack of arginine to lysine esters varied from preparation to preparation (9). Preliminary heat denaturation studies had suggested the presence of two enzyme precursors; the precursor of an enzyme capable of attacking LEe alone, along with the precursor of an enzyme capable of attacking both TAMe and LEe. Additional evidence for two factors in plasminogen preparations is as follows.
Activation Studies—Activation of plasminogen preparations by varying concentrations of SK revealed that the LEe and TAMe esterase activities bear different relations to the SK concentration (Fig. 2).

Heat Denaturation of Plasminogen Preparations—Heat denaturation studies of plasminogen preparations at selected pH values revealed different stabilities for the precursors of the LEe esterase and TAMe esterase (Fig. 3). Shown in Fig. 3 are heat denaturation studies of a plasminogen preparation at pH 2.0 and 9.0. The precursor of the TAMe esterase was more stable at pH 9.0, whereas the situation was reversed at pH 2.0. Heating plasminogen to 100° at pH 2 for prolonged periods resulted, upon activation, in an ever increasing ratio of LEe esterase to TAMe esterase activity. Occasionally, after 90 per cent or more of the initial activity was destroyed, preparations were obtained which, upon activation, contained only LEe esterase activity. These observations suggest the presence of the precursor of an enzyme with its activity limited to lysine substrates. Support for this view was obtained when fresh SK was added to heated plasmin preparations (Table III) which resulted in an increase in LEe esterase action with no increase in the TAMe esterase. Heating plasminogen to 50° at pH 9, for periods long enough to destroy 60 per cent or more of the initial activity, resulted in preparations which, upon activation, exhibited a constant ratio of TAMe esterase to LEe esterase activity of approximately 4. These latter observations suggested the presence of the precursor of a second enzyme capable of splitting TAMe at 4 times the rate of LEe.

Heat Denaturation of Plasmin Preparations—Heat denaturation studies of SK-activated plasminogen preparations revealed different stabilities for the TAMe and LEe esterases (Fig. 4). Shown in Fig. 4 are the stabilities for the TAMe and LEe esterases at 37° in the presence of 100 and 1000 units of SK. Only the stability of the LEe esterase was related to SK concentration, the LEe esterase being more stable in the presence of 1000 units of SK than in the presence of 100 units. The TAMe esterase stability appeared to be unaffected by SK concentration.

Inhibition Studies Relating LEe Esterase to Plasminogen Activator, and TAMe Esterase to Proteolytic Enzyme Plasmin

Previous studies had demonstrated that LEe and TAMe were striking competitive inhibitors of plasmin (9). In the following studies, the inhibition of the SK activation of plasminogen preparations by these synthetic substrates was investigated. The method used was to measure and compare the inhibition of enzyme activity when the synthetic substrate was added before and after SK activation. Inhibition of activation was apparent when the addition of inhibitor before SK activation resulted in a greater inhibition.
Inhibition of Activation of TAME Esterase-Proteolytic Enzyme—LEe was observed to be a very striking inhibitor of activation of the TAME esterase. Activation of the proteolytic enzyme was similarly inhibited, suggesting that the TAME esterase and the enzyme responsible for casein proteolysis were identical. TAME, on the other hand, was a poor inhibitor to the activation of the enzyme splitting TAME and casein. Data in Table I compare the inhibition of the TAME esterase activity by LEe, lysine, and ornithine and arginine when these inhibitors were added before or 5 minutes after SK activation. LEe was by far the most effective inhibitor of activation. Arginine had no inhibiting effect.

Since TAME and LEe are effective competitive inhibitors in the proteo-
lytic assay (9), studies of the inhibiting effect of the synthetic substrates on the activation of the proteolytic enzyme required the removal of the esters prior to the final assay. This was accomplished by precipitating the activated enzyme with 1 M sodium chloride at pH 2, a technique which precipitated the enzyme quantitatively and left the esters in the supernatant fluid. Assays carried out in this fashion revealed that LEe inhibited the activation of the enzyme responsible for both proteolytic and TAMe esterase activity (Table I). TAMe at a concentration of 0.02 M did not

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( M )</th>
<th>Added before SK</th>
<th>Added 5 min. after SK</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEe</td>
<td>0.005</td>
<td>75.5</td>
<td>7.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.04</td>
<td>96.6</td>
<td>43.4</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.04</td>
<td>73.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.04</td>
<td>75.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Inhibitor removed before enzyme assay

<table>
<thead>
<tr>
<th>TAMe assay</th>
<th>Proteolytic assay (casein)</th>
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<tbody>
<tr>
<td>Added before SK</td>
<td>After SK</td>
</tr>
<tr>
<td>LEe</td>
<td>67</td>
</tr>
<tr>
<td>TAMe</td>
<td>0</td>
</tr>
</tbody>
</table>

inhibit the activation of the TAMe esterase or proteolytic enzyme. At higher concentrations of TAMe, however, inhibition of activation could be demonstrated and accounts in part for the "substrate inhibition" previously noted (9).

Inhibition of Activation of LEe Esterase—Neither TAMe nor LEe inhibited the activation of the LEe esterase. LEe added before or 5 minutes after SK activation yielded identical substrate titration curves. TAMe added before SK had the same inhibiting effect as when added 5 minutes after SK, indicating that TAMe acted only as a competitive inhibitor to the LEe esterase but had no effect on activation.

When the plasminogen activator was estimated by the fibrinolytic
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assay (11), the addition of LEe, lysine, and ornithine before or after the addition of SK resulted in an identical inhibition, suggesting that these substances did not interfere with the formation of the activator, yet were inhibitors to its action. The inhibition of the plasminogen activator by lysine and ornithine has been reported by Müllertz (13).

![Diagram](http://www.jbc.org/)

**Fig. 5.** Kinetics of plasmin activation. Plasminogen preparation (14 mg of nitrogen per ml.) was activated by 36, 100, and 300 units of SK per ml. at 25°C. Activation stopped at several time intervals by the precipitation technique described in the text. TAMe esterase and casein proteolytic activity are expressed in per cent of total activity available.

Similarities in behavior in these inhibition studies relate the LEe esterase to the plasminogen activator, and identify the TAMe esterase with the proteolytic enzyme. The observation that LEe is the most effective inhibitor for the activation of the TAMe esterase-proteolytic enzyme (plasmin) is additional evidence for this view. Further confirmation was obtained through observations on a spontaneously active human plasmin preparation. Following the addition of SK to this spontaneously active preparation, a marked increase in LEe esterase activity and of plasminogen

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4 Human plasma Fraction III, obtained through the courtesy of Dr. D. M. Sugenor of Harvard University.
activator (fibrinolytic assay) was observed with little change in the TAMe esterase or proteolytic activity.

**TABLE II**

*Effect of Addition, Inactivation (Precipitation Technique), and Readdition of SK on Activation of Plasmin (TAMe Esterase-Proteolytic Enzyme), and of Plasminogen Activator (LEe Esterase)*

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Plasmin assays</th>
<th>Activator assays</th>
<th>SK assay (Christensen (10)) units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TAM$_{e}$, amole ester hydrolyzed</td>
<td>LE$_{e}$, amole ester hydrolyzed</td>
<td>Fibrinolytic units</td>
</tr>
<tr>
<td>I. Plasminogen</td>
<td>1.0</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>II. (I) + SK</td>
<td>10.3</td>
<td>3.9</td>
<td>10.0</td>
</tr>
<tr>
<td>III. Ppt. of (II) with 1 M NaCl, pH 2</td>
<td>9.4</td>
<td>3.3</td>
<td>3.1</td>
</tr>
<tr>
<td>IV. (III) + SK</td>
<td>7.6</td>
<td>2.6</td>
<td>8.9</td>
</tr>
<tr>
<td>V. Ppt. of (IV) with 1 M NaCl, pH 2</td>
<td>4.5</td>
<td>1.6</td>
<td>4.2</td>
</tr>
<tr>
<td>VI. (V) + SK</td>
<td>3.9</td>
<td>1.3</td>
<td>7.9</td>
</tr>
</tbody>
</table>

* Remmert and Cohen (12).
† Christensen (10).

**TABLE III**

*Effect of Addition, Inactivation (Heating Technique), and Readdition of SK on Activation of Plasmin (TAMe Esterase-Proteolytic Enzyme), and of Plasminogen Activator (LEe Esterase)*

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Plasmin assays</th>
<th>Activator assays</th>
<th>SK assay (Christensen (10)) units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TAM$_{e}$, amole ester hydrolyzed</td>
<td>LE$_{e}$, amole ester hydrolyzed</td>
<td>Fibrinolytic units</td>
</tr>
<tr>
<td>I. Plasminogen</td>
<td>2.5</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>II. (I) + SK</td>
<td>12.4</td>
<td>3.6</td>
<td>19.4</td>
</tr>
<tr>
<td>III. (II) heated to 100° for 15 min., pH 2</td>
<td>7.1</td>
<td>3.0</td>
<td>1.6</td>
</tr>
<tr>
<td>IV. (III) + SK</td>
<td>6.3</td>
<td>2.3</td>
<td>9.0</td>
</tr>
</tbody>
</table>

*Mechanism of Activation of Plasmin (TAM$_{e}$ Esterase) and of Plasminogen Activator (LE$_{e}$ Esterase)*

The true kinetics of plasminogen conversion to plasmin, following the addition of SK, were not observed unless the activation was stopped by removal of SK prior to estimation of plasmin activity. This was accomplished by a 2-fold reprecipitation of the activated plasmin at pH 2 with 1 M sodium chloride, a procedure which inactivated SK and stopped further
activation. By using this procedure at several time intervals, first order kinetics were observed for the activation of plasmin as measured either by the TAME esterase or the casein assay (Fig. 5), and are in agreement with the observation of Christensen (3).

The kinetics of activation of plasminogen activator could not be studied in these experiments, since its activity, when measured either by the LEE esterase or the fibrinolytic assay, required the presence of SK. The dependence of LEE esterase and fibrinolytic assay activity on the presence of SK was demonstrated by experiments involving the removal of previously added SK, followed by the fresh addition of SK. From the data shown in Tables II and III, removal of SK, either by heating or precipitation, caused a prompt fall in plasminogen activator, while the addition of fresh SK produced an immediate rise of this enzyme. No such effect was noted on the TAME esterase-proteolytic enzyme (plasmin). The activation of plasminogen activator appears to be instantaneous. The amount of activator formed and the stability of the activator (Fig. 4) were observed to be a function of SK concentration. Our observations with the fibrinolytic assay are in agreement with those of Wasserman (5).

It is concluded from these activation experiments that plasmin (TAME esterase) is activated in enzymatic fashion, whereas the plasminogen activator (LEE esterase) is reversibly activated by SK in stoichiometric fashion.

**DISCUSSION**

The following schemes, similar to that proposed by Müllertz and Lassen (7), summarize the conclusions of our observations:

\[
\text{Plasminogen proactivator + SK } \xrightarrow{\text{SK}} \text{ plasminogen activator (LEE esterase)}
\]

\[
\text{Plasminogen } \xrightarrow{\text{activator}} \text{ plasmin (TAME esterase-proteolytic enzyme)}
\]

Our work, by demonstrating the reversibility of this reaction, has provided new evidence for the stoichiometric interaction of SK with a plasma factor to form a plasminogen activator. Two possibilities remain to explain this interaction. SK may remove an inhibitor, or it may itself combine with the proactivator to form the activator.

In the enzymatic conversion of plasminogen to plasmin, we have confirmed the first order kinetics of the conversion. Identification of the enzymatic agent in this conversion as an LEE esterase is suggested by demonstration of the inhibition of plasminogen activation by LEE.

The enzymatic activation of plasminogen by plasminogen activator bears some similarity to the activation of trypsinogen by trypsin. The
activation of trypsinogen by trypsin has been shown to involve the splitting of a lysine peptide bond (14). The identification of plasminogen activator as an enzyme capable of splitting only lysine esters suggests that the activation of plasminogen also involves the splitting of a lysine peptide bond. It is of interest that trypsin can activate both human and bovine plasminogen (15).

The variability in resistance of different animal plasminogens to SK activation is well known (16). Bovine plasminogen is completely resistant to SK activation (16). Recent studies relate this lack of activation to the inability of SK to form a plasminogen activator in bovine plasma or bovine plasminogen preparations. In the presence of traces of human plasma or human plasminogen preparations, SK readily "activates" bovine as well as other animal plasminogens (7, 11). Since this latter phenomenon occurs in the fibrinolytic assay, in which the bovine fibrinogen substrate is heavily contaminated with plasminogen, a measure of the human plasminogen activator rather than the plasmin is obtained. It should be emphasized, however, that the clot is dissolved by bovine plasmin, which is the fibrinolytic enzyme, and not by the activator being measured.

The observation of the presence in human plasma of the precursor of an enzyme capable of rapidly activating plasminogen suggests that this substance may bear a relation to the natural mechanism of plasminogen activation.

**SUMMARY**

The activation of human plasminogen by streptokinase involves two steps: the "stoichiometric" interaction of streptokinase with a plasma factor to form an activator and the enzymatic activation of plasminogen by this activator. The plasminogen activator has the properties of an enzyme splitting lysine esters alone, and plasmin is a proteolytic enzyme capable of splitting arginine and lysine esters.

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**BIBLIOGRAPHY**

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