Proactivator Function of Human Plasmin as Shown by Lysine Esterase Assay*†

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Tillett and Garner (1) discovered that an extracellular protein elaborated by certain strains of streptococci produces the lysis of clots which contain human fibrinogen or thrombin. Milestone demonstrated that the bacterial product was not in itself fibrinolytic but interacted with a lytic material in human plasma (2). The situation was greatly clarified and a rational nomenclature introduced by Christensen (3), who showed that the bacterial protein, streptokinase, activated the lytic precursor, plasminogen, to a proteolytic enzyme, plasmin.

The concept that streptokinase acts directly on plasminogen to catalyze its conversion to plasmin has given way to the theory that this conversion is a two-step reaction. Streptokinase, which exhibits little activity in any species other than the human, first reacts stoichiometrically with a precursor in human plasma to form a plasminogen activator which then enzymatically causes the transformation of plasminogen to plasmin in virtually all species (4-6). The nature of the precursor has been a matter of considerable controversy. Because of its inseparability from human plasminogen under a variety of conditions, the precursor has been claimed to be plasminogen itself (7-9). Other investigators, to avoid assigning two distinct functions to the same plasminogen molecule, have proposed that the precursor is a unique protein which they called "proactivator" (5). The evidence in this controversy has been recently reviewed (10).

Methods for the measurement of proactivator and activator activities have depended on the estimation of plasmin evolved from bovine plasminogen. The production of plasmin to measure these activities involves serious limitations since preformed plasmin or the presence of plasmin inhibitors interferes. In this communication, assays are proposed for the quantitative estimation of proactivator, activator, plasminogen, and plasmin in the presence of each other, based on the hydrolysis of lysine methyl ester. Both plasmin and the activator hydrolyze lysine methyl ester, but evidence will be presented indicating that only the former is inhibited by soybean trypsin inhibitor. The residual activity, therefore, is a measure of the activator. Conditions for the lysine methyl ester assay of plasminogen and of the precursor of the activator have also been defined.

Since the various activities can be estimated in the presence of each other, it was possible to study the interconversion of components of the streptokinase-induced fibrinolytic system. The findings strongly point to plasmin as a precursor which reacts with streptokinase to form a plasminogen activator. The possibility that a plasminogen-streptokinase complex may also have activator activity cannot be excluded at this time although tests have given negative results. These results also do not exclude the possibility that other proactivators may exist in human blood.

EXPERIMENTAL PROCEDURE

Plasminogen was prepared from human Fraction III by a previously published method (11). The Fraction III was obtained from E. R. Squibb and Sons through the courtesy of the American National Red Cross. Activator solutions were prepared by the addition of 5000 units of streptokinase to each milligram of plasminogen and subsequent alcohol fractionation (12). These solutions also contained plasmin (7). Plasmin solutions were prepared by salt fractionation after the activation of purified plasminogen with small amounts of streptokinase. Tests for activator or streptokinase activities were negative with casein, LME, and fibrin plate substrates (13).

Lysine methyl ester dihydrochloric acid was obtained from the Mann Research Laboratories, Inc. Streptokinase (Varidase; Lederle Laboratories) was generously supplied by Dr. E. C. de Renzo. Soybean trypsin inhibitor, "antitrypsin soy," Parke, Davis and Company, was employed.

The hydrolysis of LME was followed by the procedure of Hagan, Ablondi and Hutchings (14). Incubation time was shortened to 30 minutes. Casein was purified by the method of Mullertz (15). The procedure used for caseinolytic assay was a modification of that of Remmert and Cohen (16). The solution to be measured plus activator or inhibitor was made up to 3 ml with 0.1 M phosphate buffer, pH 7.4. Three milliliters of 3% casein in phosphate buffer were added and the reaction mixture was then added to 3 ml of 11.5% trichloroacetic acid and the precipitate was agglomerated by incubation at 37° for 30 minutes. The supernatant solution, containing trichloroacetic acid-soluble material, was obtained by centrifugation followed by filtration through a small plug of glass wool. Absorption at 280 mμ was read in a Beckman model DU spectrophotometer, and the results were expressed as caseinolytic units by reference to a standard tyrosine curve. Each unit represents the liberation of 450 μg of tyrosine equivalents per hour.

RESULTS

Validity of Lysine Esterase Assay—As a prerequisite to the utilization of LME hydrolysis in the estimation of activator and

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† The abbreviation used is: LME, lysine methyl ester.
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Fig. 1. LME hydrolysis by plasmin and activator showing soybean trypsin inhibitor, SBTI, inhibition of plasmin activity. Top curve, plasmin plus activator; bottom curve, plasmin. Activator solution (see “Experimental Procedure”) contained plasmin as well as activator. The volume which produced an optical density of 0.4 in the LME assay was determined and residual LME activity was measured in the presence of the amounts of soybean trypsin inhibitor shown. Plasmin (see “Experimental Procedure”) was measured similarly.

Fig. 2. The inhibition of LME hydrolysis by soybean trypsin inhibitor, SBTI, in solution made by the addition of 25,000, 10,000, and 5,000 units of streptokinase per mg of plasminogen. Residual LME activity represents activator activity and correlates with the amount of streptokinase used. The volume of activator solution to produce an optical density of from 0.3 to 0.4 in the LME assay was determined and residual LME activity was measured in the presence of the amounts of soybean trypsin inhibitor shown.

Plasmin activities, it was necessary to show that soybean trypsin inhibitor specifically inhibits plasmin without affecting activator activity. Solutions of activator plus plasmin were compared with solutions of plasmin alone, after the addition of increasing concentrations of inhibitor. As the inhibitor concentration increased, the values obtained in casein assays decreased, indicating that plasmin was being inhibited. LME splitting by the activator-free solution was completely inhibited, but the curve of the activator-rich solution reached a plateau (Fig. 1), supporting the idea that soybean trypsin inhibitor inhibits plasmin but does not block LME hydrolysis by the activator. The plateau portion of the curve shows that a 5-fold increase in inhibitor concentration did not diminish the hydrolysis of LME by the activator. The presence of soybean trypsin inhibitor does not interfere in fibrin plate assays. In such tests, curves similar to those found in LME assays were obtained.

Activator solutions of different strengths were prepared by the addition of increasing amounts of streptokinase to a fixed volume of precursor solution. The assay of activator (LME hydrolysis) was carried out in the presence of varying amounts of soybean trypsin inhibitor. The residual activity after the plateaus were reached, presumably indicating activator activity, reflected the amount of streptokinase added, and, therefore, the amount of activator formed (Fig. 2). When a standard curve was run with increasing concentrations of a single activator solution, a straight line was obtained (Fig. 3).

The precursor of the activator may also be measured by LME hydrolysis in the presence of soybean trypsin inhibitor provided that the amount of streptokinase used is properly controlled. The measurement of this activity is complicated by the fact that two products are formed when streptokinase is added to a human euglobulin or purified plasminogen preparation: plasmin and the activator. The amount of each produced depends on the ratio of streptokinase to precursor (see Fig. 3). To standardize the relative amounts of activator and plasmin formed, the plasminogen content of the unknown solution was first determined in casein assays. Even if the proactivator is not plasminogen, the ratio of these two activities has been shown to be identical in unpurified and highly purified preparations (17). With a fixed ratio of streptokinase to precursor, 2000 streptokinase units per casein unit, a standard curve was established by measurement of LME hydrolysis in the presence of soybean trypsin inhibitor (Fig. 4). Twice the quantity of inhibitor necessary to produce complete plasmin inhibition was used.

Fig. 3. Standard activator curve. LME hydrolysis in the presence of 1 mg of soybean trypsin inhibitor. An optical density of 0.4 in the LME assay was produced by 0.4 ml of activator solution; 0.5 mg of soybean trypsin inhibitor completely inhibited the plasmin in this solution. Twice this amount, 1 mg of soybean trypsin inhibitor per assay, was used.
This arbitrarily selected ratio of streptokinase to precursor did not produce maximal activator formation. If this procedure were to be used in other laboratories with different streptokinase to precursor ratios, proactivator values would not be comparable. Accordingly, the curve obtained when the ratio of streptokinase to precursor was increased up to 20,000 streptokinase units per casein unit (equal approximately to 200,000 streptokinase units per mg of purified plasminogen) was determined (Fig. 5). With two different preparations, above a ratio of 8,000, a straight line was obtained, and the values with and without soybean trypsin inhibitor were virtually the same; this shows the almost complete absence of plasmin activity under these conditions. It is apparent that the proactivator content of an unknown solution may be estimated by LME hydrolysis after the addition of 10,000 streptokinase units per casein unit without the addition of inhibitor. Use of the inhibitor is recommended whenever the presence of significant quantities of preformed plasmin is suspected. Lima bean trypsin inhibitor gave results similar to those obtained with the soybean inhibitor but pancreatic trypsin inhibitor (a gift from Dr. Jean Dormont, Centre National de Transfusion Sanguine, Paris) showed a much stronger affinity for plasmin and, at higher concentrations, inhibited the activator also.

The possibility that soybean trypsin inhibitor acts upon streptokinase as well as upon plasmin, blocking the appearance of activator until the concentration of streptokinase overwhelms this inhibition, was investigated and ruled out. An increase in inhibitor to 20 times the concentration used in these experiments did not alter the results appreciably.

A rigorous check of the validity of the LME assays is offered by a comparison of activator and plasmin activities obtained by means of LME analysis with independent measurements of activator by clot lysis and of plasmin by casein hydrolysis.

The activator curve obtained from LME hydrolysis in the presence of soybean trypsin inhibitor was compared with a curve obtained according to the excellent activator assay of Lassen (18) which is based on clot lysis. In Fig. 6, the values from clot lysis measurements (open circles), obtained with the use of 40 to 20,000 streptokinase units per casein unit, are superimposed on the LME curve. The agreement was highly satisfactory.

Fig. 7 shows a comparison of plasmin curves obtained with casein assays and the LME assay over the range of streptokinase concentrations from 40 to 20,000 units per casein unit. The observation of Markus and Ambrus (19) that tosylarginine methyl esterase activity develops more slowly than caseinolytic activity led us to incubate the activated solutions for 10 minutes at 37°C before assay. The rising and plateau portions of the curves coincided; this shows that within the range of streptokinase concentrations usually used, the LME method agreed with the standard caseinolytic assay. At high streptokinase levels, caseinolytic activity decreased but still persisted, whereas the inhibitable LME activity (plasmin) fell to zero. Norman (9), using casein assays, also has demonstrated a decrease in plasmin at high streptokinase levels.

It seemed possible that the caseinolytic activity observed at high streptokinase levels was not the result of plasmin activity but was due to proteolytic activity of the activator (20). Two types of experiments indicate that this surmise is correct: (a) soybean trypsin inhibitor did not inhibit this activity, and (b) casein hydrolysis was measured at ratios of streptokinase to precursor from 40 to 20,000 streptokinase units per casein unit.
Evidence That Human Plasmin Has Proactivator Potential

Formation of Activator Instead of Plasmin with Increasing Streptokinase—The addition of streptokinase to plasminogen results in the appearance of both plasmin and plasminogen activator (6). The relative amounts of each, formed as increasing amounts of streptokinase were added to a fixed amount of plasminogen, were measured. In a series of tubes, streptokinase was added to portions of the plasminogen solution to obtain increasing ratios from 40 to 20,000 streptokinase units per casein unit. The amounts of plasmin and activator formed were measured by LME hydrolysis in the presence and absence of soybean trypsin inhibitor (Fig. 9). Total LME hydrolysis, indicating plasmin plus activator activities, rose until a ratio of 750 was attained and then remained at a nearly constant level. Activator formation measured by LME activity in the presence of inhibitor, remained low until the ratio reached 100. The curve then rose, reaching a maximum at 8000. Plasmin activity, the difference between these curves, rose and then fell to zero, a plateau being evident between 75 and 1000 streptokinase units per casein unit. At the highest streptokinase levels, little or no plasmin activity was found. The rise in activator formation at high streptokinase levels was accompanied by a mirror-image fall in plasmin, strongly suggesting that streptokinase had combined directly with plasminogen to form the activator or that plasminogen was first activated to plasmin and formation of the activator followed.

Formation of Plasminogen Activator from Plasmin—Plasmin solutions were prepared by the addition of small amounts of streptokinase (100 units per casein unit) to purified plasminogen. Tests for unreacted plasminogen in these solutions were negative. The amount of activator produced was measured (see Fig. 6). The solutions were then incubated at 37° to produce gradual inactivation of the plasmin. At zero time and at intervals during the next 90 minutes, four portions were removed. Two were used in the assay for plasmin by measurement of LME hydrolysis in the presence and absence of soybean trypsin inhibitor, the difference representing plasmin, and the other two samples were treated with 10,000 streptokinase units per casein unit to convert all proactivator present to activator which was estimated by LME hydrolysis in the presence and absence of inhibitor. Upon the addition of large amounts of streptokinase, plasmin activity disappeared and activator activity appeared in its place (Fig. 10). The inactivation curve of plasmin paralleled the decrease in proactivator activity. Since glycerol stabilizes plasmin (21), the experiment was repeated in the presence of 50% glycerol (Fig. 10). Plasmin activity remained relatively stable during the 90 minutes of observation, and the addition of streptokinase to portions of the solution showed that proactivator activity had also remained near the starting level.

Formation of Plasmin from Plasminogen Activator—The conversion of activator to plasmin by acid destruction of the streptokinase activator.
kinase portion of the activator molecule has been suggested by Zylber, Blatt, and Jensen (22). In our experiments, activator was prepared by the addition of 10,000 units of streptokinase per casein unit of precursor. Upon acidification to pH 2 for 15 minutes and renaturation, casein and LME assays showed that activator activity had decreased and plasmin activity had increased (Table I). Recovery of plasmin from activator was not complete because of acid destruction of plasmin itself and failure of this treatment to inactivate the activator completely (23).

Proactivator Levels in Glycerol-activated Plasminogen Solutions—In the presence of glycerol, plasminogen is slowly converted to plasmin (21). Solutions containing plasminogen and glycerol were incubated at 37°C for 5 days. Samples were analyzed daily for preformed plasmin, plasminogen, and proactivator by LME hydrolysis in the presence and absence of soybean trypsin inhibitor. Small amounts of streptokinase were added to determine plasminogen plus plasmin and large additions of streptokinase were used to measure the amount of precursor available to form the activator. Plasminogen disappeared and was no longer detectable by the 5th day (Fig. 11). Plasmin concentration concurrently increased. Proactivator activity was as high on the 1st day when plasminogen constituted the chief activity as on the 5th day when plasmin activity was dominant. Since streptokinase is added in the proactivator assay, any plasminogen present could be converted to plasmin and then to the activator. Ablondi and Hagan (8) in a similar experiment, reported a fall in proactivator activity. In repeated experiments in which casein and clot lysis as well as LME assays were used, we have not been able to confirm their result.

The demonstration that the plasminogen activator may be formed from plasmin by the addition of streptokinase does not rule out the possibility that an activator may also exist in the form of a plasminogen-streptokinase complex. However, comparison of the activator activity resulting from the addition of large amounts of streptokinase to plasminogen and to plasmin have failed to reveal any difference in the behavior of the activator(s) formed toward casein, LME, or bovine clots.

**DISCUSSION**

Activator assays have been based on the conversion of an excess of bovine plasminogen to plasmin. The end point has been measured by clot lysis (18), esterolysis (8), or proteolysis (15). The LME assay of activator offers several advantages over current tests. (a) It is simple to carry out and does not involve the use of impure and variable clotting components. It is, therefore, reproducible and easily controlled. (b) Since the measurement is a characteristic of the activator itself, the test is independent of the production of plasmin, permitting the estimation of activator in the presence of high plasmin concentrations. (c) The formation of the activator can be measured in systems in which plasmin activity is suppressed, allowing less complicated study of the kinetics of activator formation. (d) The splitting of LME by plasmin and by the activator is in the same order of magnitude.

**Table I**

Appearance of plasmin after acidification of activator

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<th>Assay</th>
<th>Before acidification</th>
<th>After acidification</th>
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<tbody>
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<td></td>
<td>Activator</td>
<td>Plasmin</td>
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<tr>
<td>LME</td>
<td>0.296</td>
<td>0.042</td>
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<tr>
<td></td>
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<td>Casein</td>
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<td>0.055</td>
<td>0.107</td>
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</tbody>
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* Corrected for destruction of plasmin by acidification as determined in simultaneous control experiments.
magnitude, in contrast to clot tests in which the activator assay is far more sensitive than that of plasmin, permitting more precise quantitative balance studies to be made in the LME system. Disadvantages of the assay are its insensitivity relative to clot assays and the fact that all LME esterase activity induced by streptokinase may not, in unpurified systems, be the result of activator plus plasmin activity alone.

Assays for the precursor of the activator have been unsatisfactory. In clot tests, the large amount of streptokinase, which is required for maximal activator formation, inhibits clot lysis (7). Therefore, such tests are carried out with a concentration of streptokinase which is optimal for clot lysis but not for activator formation. Esterolytic and proteolytic tests cannot be used to measure weak proactivator activity in the presence of plasmin and have been found to be erratic, possibly because of the large amount of bovine plasminogen used in the system. None of these procedures is valid in the presence of plasmin inhibitors. The preliminary determination of precursor concentration by proteolytic measurement, and the use of 10,000 streptokinase units per casein unit in the LME assay, permit the accurate determination of proactivator activity after the complete conversion of precursor to activator.

Comparison of the LME assay for plasmin with the standard casein test reveals that the two measurements coincide except at high streptokinase levels where the hitherto unsuspected ability of the activator to hydrolyze casein introduced errors into the assay. Although the activator has not previously been shown to possess caseinolytic activity, the conversion of plasminogen to plasmin is known to involve the splitting of peptide bonds (20). In the usual activation studies, any caseolytic activity observed would be attributed to the plasmin which is also formed.

Upon the addition of streptokinase to plasmin, the plasmin activity disappeared and was replaced by an equivalent amount of activator activity. When an activator solution was acidified to destroy the streptokinase portion of the activator complex, activator disappeared and plasmin activity appeared. In addition, at 77°, proactivator disappearance paralleled plasmin inactivation, and stabilization of the plasmin with glyceral also stabilized the proactivator. Furthermore, proactivator activity was found to correlate with the sum of plasminogen plus plasmin activities when plasminogen was incubated with glyceral for 5 days. These results demonstrate a close relationship between plasmin and the activator and strongly suggest that human plasmin has proactivator potential.

The idea that the plasminogen activator may be a plasmin-streptokinase complex has been suggested by several authors (6, 7, 9, 22) but convincing evidence has been lacking. An important consequence of this theory requires that if plasmin is the only streptokinase-activated proactivator, some plasmin must be present to initiate the reaction. It is interesting that the most highly purified plasminogen preparations contain spontaneous plasmin activity (17). The additional possibility that streptokinase can react directly with plasminogen to form an activator cannot be excluded. However, attempts to demonstrate two activators produced by the addition of streptokinase to plasminogen and to plasmin, respectively, by the effect on three different substrates, casein, LME and bovine clots, were unsuccessful. The ability of plasmin to function as a proactivator eliminates the necessity, required by a plasminogen-streptokinase hypothesis, to assign two functions to the plasminogen molecule.

It is important to note that the proactivator potentiality of plasmin does not eliminate the possibility that another proactivator may exist in human blood. Evidence indicating that a second proactivator distinct from plasminogen or plasmin may exist in human plasma has been reported by Markus and Werkheiser (24) and had been found by Dr. N. Spritz and by us.

SUMMARY

1. A method for the estimation of plasmin and the activator of plasminogen by means of lysine methyl ester hydrolysis, with and without soybean trypsin inhibitor, is described. Measurement of the activator by its esterolytic activity permits its estimation in the presence of plasmin.

2. Conditions for activation of the proactivator by the same technique are defined.

3. The activator was found to possess caseinolytic activity.

4. Evidence is presented from studies of the formation of activator from plasminogen, plasmin, or mixtures of the two, that human plasmin can serve as a precursor of the plasminogen activator which is formed by the addition of streptokinase to plasminogen solutions. Stability studies and the demonstration of the interconversion of plasmin and the plasminogen activator by the addition of streptokinase to the former or removal of streptokinase from the latter, strongly support this hypothesis.

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The talk given deviated from the abstract.

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