Kinetic Mechanism of the Activation of Human Plasminogen by Streptokinase†

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ABSTRACT: A method of determining the initial rate of plasminogen activation has been developed. The method has been used to investigate the mechanism of activation of human plasminogen by streptokinase. Plasmin formation follows saturation kinetics. Inhibition of plasmin formation by ϵ-aminocaproic acid is uncompetitive with a K_i of 0.6 mM. A model consistent with the data is that streptokinase induces a conformational change in the plasminogen molecule, producing an active center which cleaves an internal peptide bond to produce plasmin. Thus, streptokinase functions as a catalytic allosteric effector.

The kinetic mechanism of the activation of human plasminogen to plasmin by the bacterial protein streptokinase is obscure. Streptokinase activation of plasminogen occurs via hydrolytic cleavage of an arginyl-valyl bond (Summaria et al., 1967); however, streptokinase cannot hydrolyze synthetic esters of arginine or lysine (DeRenzo et al., 1967a) nor can it be titrated with the active center-specific reagent, *p*-nitrophenyl-*p'*-guanidinobenzoate (McClintock and Bell, 1971). When streptokinase and human plasminogen are incubated together a 1:1 complex is formed which contains an active site titratable by *p*-nitrophenyl-*p'*-guanidinobenzoate or *p*-nitrophenyl-*p'*-(amidinothiomethyl)benzoate and this active center appears before cleavage of the peptide chain of plasminogen (McClintock and Bell, 1971; Reddy and Markus, 1972; Schick and Castellino, 1974). It is well established that bovine plasminogen is not activated by streptokinase alone and requires the presence of both human plasminogen and streptokinase. This observation has led to the proposal that bovine plasminogen is activated by a stoichiometric complex of human plasmin and streptokinase (Kline and Fishman, 1961; Markus and Werkheiser, 1964; Troll and Sherry, 1955; Werkheiser and Markus, 1964). By analogy it has been assumed that human plasminogen must also be activated by a streptokinase–human plasminogen or streptokinase–human plasmin complex. Data to support this assumption have been obtained by indirect means such as using inhibitors and alternate substrates to demonstrate that the streptokinase/plasminogen ratio alters the esterolytic and caseinolytic characteristics of the mixture (Kline and Fishman, 1961; Reddy and Markus, 1972, 1974; Markus and Werkheiser, 1964). The ability to isolate a stoichiometric complex of plasmin and streptokinase in the presence of the plasmin inhibitor ϵ-aminocaproic acid (Davies et al., 1964; DeRenzo et al., 1967b) is also cited as evidence for the role of a stoichiometric complex in the activation of human plasminogen. The decreased caseinolytic activity of plasmin in the presence of large amounts of streptokinase may be explained by alternate substrate inhibition since plasmin is known to cleave various peptide bonds of streptokinase (Brockway and Castellino, 1974; McClintock et al., 1974; Taylor and Beisswenger, 1973) and the ability to isolate a streptokinase–plasmin complex is analogous to the isolation of an E–P complex. Thus the question of whether streptokinase itself activates human plasminogen or whether a streptokinase–plasmin or streptokinase–plasminogen complex is the species which activates a second molecule of plasminogen to plasmin has not been unequivocally answered. This paper presents a means for measuring the initial rate of plasmin formation from human plasminogen. Using this assay for the reaction catalyzed by streptokinase, we have demonstrated that plots of the reciprocal of velocity vs. reciprocal of plasminogen concentration are linear and thus the generation of plasmin does not require that 2 mol of plasminogen react per mol of streptokinase. Rate assays of this type should have general applicability to the study of reactions which generate an active enzyme species from a proenzyme.

Experimental Procedure

Human plasminogen was prepared from normal human plasma by the following modification of the affinity chromatography method of Deutsch and Mertz (1970). After the plasma was applied to the Sepharose-lysine column, the column was washed with 3 mM phosphate buffer (pH 7.0) containing 2 mM EDTA until the A_{280} decreased to less than 0.1. The column was then washed with the same buffer containing 1 M NaCl until the nonspecifically absorbed protein was eluted. Silverstein (1974) has recently reported that NaCl is more efficient than phosphate for eluting these contaminants from the column. The plasminogen was then eluted with 0.2 M ϵ-aminocaproic acid containing 2 mM EDTA and 3 mM phosphate buffer (pH 7.0). The plasminogen was precipitated by the addition of 0.32 g of $(NH_4)_2SO_4$/ml. The precipitate was dissolved in 20 mM Tris-Cl (pH 7.5)–2 mM EDTA, dialyzed against the same buffer, and stored in liquid nitrogen.

Electrophoresis of reduced samples of plasminogen on 7.5% polyacrylamide gels containing 1% dithioerythritol and 1% sodium dodecyl sulfate as described by Wiman and Wallen (1973) gave one major band with only a trace of contaminating protein when stained with Coomassie Blue. When the plasminogen was activated with catalytic amounts of streptokinase, the electrophoretograms showed that over 90% of the plasminogen was activated as judged

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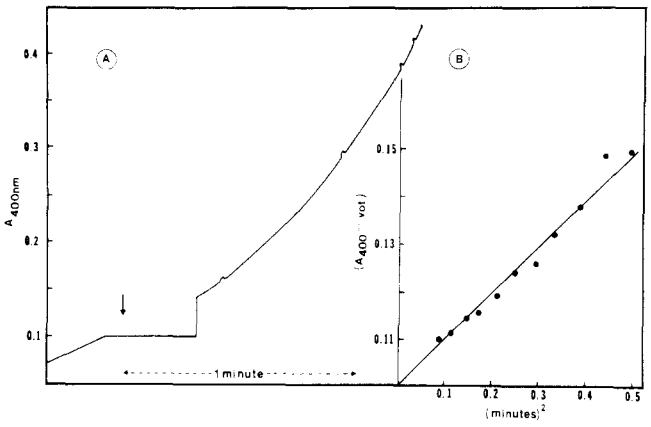

FIGURE 1: Assay of production of plasmin. Cuvet contained Tris-HCl (pH 7.0), 0.1 *M*; *N*-α-Cbz-lysine-*p*-nitrophenyl ester, 2×10^{-4} *M*; plasminogen, 28 μg. At the arrow 1.1 μg of streptokinase was added. The final volume was 1 ml. The ester was added as an acetone solution, therefore, acetone was always present at a concentration of 1%. (A) Tracing of the chart obtained at a speed of 5 in./min and a full-scale deflection equivalent to an optical density of 0.5; (B) replot of the data as described in the text.

by the disappearance of the plasminogen band and the appearance of the heavy and light chains of plasmin. The purity of the plasminogen was also determined by the active site titration method of Chase and Shaw (1967, 1969) using a Cary double beam spectrophotometer. The sample cuvet contained 0.1 *M* triethanolamine-HCl (pH 8.1) and 0.1 *M* L-lysine, 200–400 μg of plasminogen, and 5–13 μg of streptokinase in a final volume of 1 ml. The reference cuvet contained only buffer and lysine. After 30-min incubation at 30°, *p*-nitrophenyl-*p'*-guanidinobenzoate was added at a final concentration of 10^{-5} *M* to both cuvets. The burst hydrolysis was measured at 410 nm and corrected for post-burst hydrolysis by extrapolation to zero time. Assuming a molecular weight of 87,000 determined by dodecyl sulfate gel electrophoresis described above (this is the same value reported by Walther et al. (1974)) the plasminogen was 85–89% active by this method. When fully activated by streptokinase, 10 μg of this plasminogen hydrolyzed 10 nmol of *N*-α-Cbz-*p*-nitrophenyl ester[1] per min at 30° and pH 7.0. This gives a k_{cat} of 2/sec compared to the k_{cat} of 24/sec reported by Silverstein (1973) at pH 6.0 and room temperature. The reason for the lower k_{cat} of our plasminogen may be due to the different assay conditions, since by all other criteria the plasminogen is at least 85% pure.

Streptokinase was provided by Dr. W. L. Warner of Hoechst-Roussel Pharmaceuticals, Inc. The preparation showed only a single band of 44,000 daltons on 7.5% polyacrylamide–1% dodecyl sulfate electrophoretograms. Normal human plasma was supplied by the Washington, D. C., Blood Center of the American National Red Cross. *N*-Benzoylphenylalanylvalylarginyl-*p*-nitroanilide was purchased from AB Bofors, Molndal, Sweden. *N*-α-Cbz-L-lysine-*p*-nitrophenyl ester was purchased from Calbiochem.

Protein concentrations were determined spectrophotometrically at 280 nm (Summaria et al., 1968). Spectrophotometric assays were performed in a Cary 118B spectrophotometer with the cell compartment maintained at 30°.

[1] Abbreviations used are: Cbz, benzyloxycarbonyl; SK, streptokinase; Plg, plasminogen; Pl, plasmin; HPlg, human plasminogen; HPl, human plasmin; BPlg, bovine plasminogen; BPl, bovine plasmin.

Results

Rate Assay for the Formation of Plasmin. The spectrophotometric rate assay for the generation of plasmin is similar to the Factor Xa generation assay of Kosow et al. (1974). The assay is based on the fact that plasmin can hydrolyze *N*-α-Cbz-lysine-*p*-nitrophenyl ester (Silverstein, 1973) and thus the rate of formation of plasmin will be proportional to the acceleration of the rate of hydrolysis of *N*-α-Cbz-lysine-*p*-nitrophenyl ester. When streptokinase, plasminogen, and Cbz-lysine-*p*-nitrophenyl ester are incubated in a recording spectrophotometer, the pen traces a parabola (Figure 1A) conforming to the equation $A_{400} = v_0 t + \frac{1}{2}at^2$ where t is time, a is acceleration, and v_0 is the endogenous rate of hydrolysis (Kosow et al., 1974). The endogenous rate is due to the spontaneous hydrolysis of the *p*-nitrophenyl ester and is dependent upon the concentration of the ester. Also the above equation is valid only while the plasmin is being produced at a constant rate. For these reasons the reaction is followed for very short times before significant amounts of either plasminogen or *p*-nitrophenyl ester are utilized. Thus both v_0 and a are constant while the reaction is being followed. Note that the above equation is analogous to the equation for rectilinear motion with constant acceleration. It follows that when $A_{400} - v_0 t$ is plotted vs. t^2, a straight line will be obtained (Figure 1B) whose slope (which equals $a/2$) is proportional to the rate of formation of plasmin. When streptokinase and plasminogen were incubated for 20 min (to allow complete activation) before addition of Cbz-lysine-*p*-nitrophenyl ester linear rates of hydrolysis were obtained. The K_m for this preformed plasmin was 3×10^{-5} *M*, the same as reported by Silverstein (1973). *N*-α-Cbz-lysine-*p*-nitrophenyl ester was present at a concentration of 2×10^{-4} *M* (seven times its K_m) in all experiments.

Due to reports that different products are formed at different streptokinase/plasminogen ratios and that at high streptokinase concentrations no free plasmin is formed (Kline and Fishman, 1961; Werkheiser and Markus, 1964) as well as the recent report that plasmin, streptokinase–plasmin, and streptokinase–plasminogen have different kinetic constants for the hydrolysis of acetyllysine methyl ester (Reddy and Markus, 1974) it is necessary for us to demonstrate that we are measuring a single reaction. Although it is difficult to envision that different products are formed at varying streptokinase/plasminogen ratios, it is possible that varying mole ratios of plasmin, streptokinase/plasmin, and streptokinase/plasminogen will be produced. In order to test the possibility that the Cbz-lysine-*p*-nitrophenyl ester was being hydrolyzed by several esterase activities we measured the K_m of the ester for the products formed after 20 min at constant plasminogen but at streptokinase/plasminogen mole ratios from 1:1 to 1:60. The results are illustrated in Figure 2. Note that there is no alteration in either the V_{max} or the K_m. This indicates that only one product is being formed and measured or that if there is more than one product being measured all the products have the same kinetic constants.

Pseudo-First-Order Kinetic Studies. Further evidence that there is only one esterolytic species being produced under our experimental conditions and that this esterase is plasmin and not merely a streptokinase–plasminogen or streptokinase–plasmin stoichiometric complex was obtained by measuring the pseudo-first-order rate constant under conditions where more moles of plasminogen are utilized than moles of streptokinase are present.

Table I: Pseudo-First-Order Rate Constants.[a]

Plasminogen (μg)	Streptokinase (μg)	Plasmin Formed in 20 min (units)[b]	k
3.5	0.55	3×10^{-3}	0.23
3.5	1.1	3×10^{-3}	0.44
10	0.55	9×10^{-3}	0.26
10	1.1	11×10^{-3}	0.60

[a] Incubation mixture was the same as in Figure 3. [b] One unit is defined as the amount of plasmin which can hydrolyze one micromole of Cbz-lysine-*p*-nitrophenyl ester per minute at 30°.

Table II: Pseudo-First-Order Rate Constants Using Benzoylphenylalanylvalylarginyl-*p*-nitroanilid.[a]

Plasminogen (μg)	Streptokinase (μg)	Plasmin Formed in 20 min (units)[b]	k
20	0.1	2.8×10^{-3}	0.046
10	0.2	1.6×10^{-3}	0.13
10	1.0	1.4×10^{-3}	0.41
20	1.0	2.8×10^{-3}	0.43
40	1.0	6.6×10^{-3}	0.35
10	2.0	1.6×10^{-3}	0.70
20	2.0	3.0×10^{-3}	0.98

[a] Reaction mixtures the same as Table I except 0.1 m*M* benzoylphenylalanylvalylarginyl-*p*-nitroanilide was substituted for the Cbz-lysine-*p*-nitrophenyl ester. [b] One unit is defined as the amount of plasmin which can hydrolyze one micromole of benzoylphenylalanylvalylarginyl-*p*-nitroanilide per minute at 30°.

McClintock and Bell (1971) have reported that the reaction of streptokinase and human plasminogen leading to a titratable active site follows first-order kinetics when the streptokinase concentration exceeds the plasminogen concentration. The first-order plots shown in Figure 3 demonstrate that when the reaction between streptokinase and plasminogen is allowed to proceed until significant amounts of plasminogen are converted to plasmin, the production of plasmin follows first-order kinetics. It can be seen from Figure 3 and Table I that the total amount of plasmin formed is directly proportional to the plasminogen added and is not related to the streptokinase concentration. The pseudo-first-order rate constant is not altered by the initial concentration of plasminogen but is directly proportional to the streptokinase concentration (Table I). When benzoylphenylalanylvalylarginyl-*p*-nitroanilide was used as the chromogenic substrate (Bergstrom and Blomback, 1974) similar results were obtained (Table II). Since the *p*-nitroanilide ester has an insignificant rate of endogenous hydrolysis, much higher ratios of plasminogen to streptokinase could be utilized than was the case when the relatively unstable *p*-nitrophenyl ester was the chromogenic substrate. It is evident that the pseudo-first-order rate constant is proportional to the streptokinase concentration over a 40-fold range of plasminogen/streptokinase ratios and that neither the plasminogen concentration nor the chromogenic substrate alter the pseudo-first-order rate constant (Tables I and II). These results are consistent with the idea that we are measuring the activation of plasminogen to plasmin catalyzed by streptokinase.

It has been proposed that when streptokinase and plasminogen combine to form a complex, an activator with esterolytic activity is formed which in a separate reaction can produce plasmin from plasminogen (Reddy and Markus, 1972,

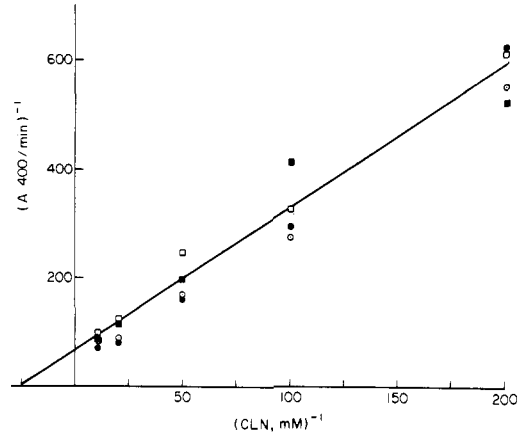

FIGURE 2: Effect of streptokinase concentration on hydrolysis of *N*-α-Cbz-lysine-*p*-nitrophenyl ester (CLN) by plasmin; 75 μg of plasminogen (0.9 nmol) was incubated 20 min in 1 ml of 0.1 *M* Tris-HCl (pH 7.0) with 36 μg (0.8 nmol, [●]); 18 μg (0.4 nmol, [○]); 1.4 μg (0.03 nmol, [■]); or 0.72 μg (0.015 nmol. [□]) of streptokinase. A 5-μl aliquot was then transferred to a cuvet containing 1 ml of 0.1 *M* Tris-HCl (pH 7.0) and from 5×10^{-3} to 0.1 m*M* CLN. Esterase activity was assayed at 400 n*M*.

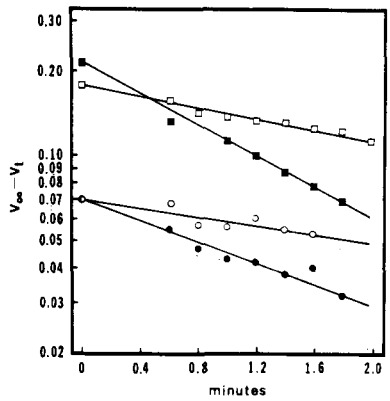

FIGURE 3: First-order plot of plasmin formation. Conditions were the same as Figure 1 except streptokinase concentration was 0.55 μg (○, □) or 1.1 μg (●, ■) and plasminogen concentration was 3.5 μg (○, ●) or 10 μg (□, ■). Velocity units are A_{400}/min. V_∞ is the maximum velocity achieved and was determined in a separate tube which was incubated for 20 min before the addition of Cbz-lysine-*p*-nitrophenyl ester. V_t is the velocity at the time indicated on the abscissa.

1973). It could be argued that the rate assay, used to measure initial rates of plasmin formation, is merely measuring the production of the activator and not plasmin formation. Since the first-order plots in Figure 3 are linear, only one reaction is being measured. Plasminogen is about twice the molecular weight of streptokinase, thus in the reaction mixtures containing 10 μg of plasminogen (Figure 3, □, ■), the plasminogen is present in about fivefold (Figure 3, ■) or tenfold (Figure 3, □) molar excess. As the reaction was allowed to proceed until at least half of the plasminogen was utilized, more moles of plasmin were produced than moles of streptokinase were present, thus, it is impossible for the measured reaction to be stoichiometric. Furthermore, if the reaction were stoichiometric, the kinetics would be second order and the pseudo-first-order rate constant would increase as the plasminogen concentration increases. As shown in Tables I and II the data do not conform to a second-order reaction. If there were a rapid formation of activator which was complete before the first observation, the extrapolated V_∞ would be less than the true V_∞ measured

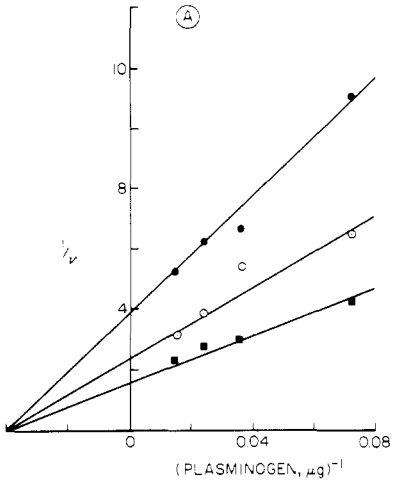

FIGURE 4: Initial velocity of plasmin formation. Reaction conditions were the same as in Figure 1 except plasminogen concentrations were varied and streptokinase was 0.55 μg (●), 1.12 μg (○), or 1.68 μg (■). Velocity units are A_{400}/min^2.

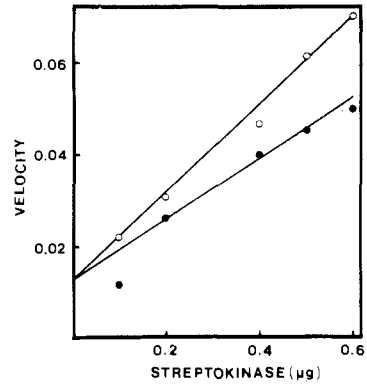

FIGURE 5: Initial velocity of plasmin formation catalyzed by streptokinase (○) or streptokinase–plasmin (●); 10 μg of streptokinase was incubated 20 min at 30° in 0.1 *M* Tris-Cl in the presence (●) or absence (○) of 100 μg of plasminogen. Final volume was 1 ml. Aliquots of these solutions to give the desired amount of streptokinase were then used for the activation of plasminogen as described in Figure 1. Each cuvet contained 50 μg of plasminogen and the data were corrected for the hydrolytic activity due to the plasmin produced in the first incubation. Velocity units are A_{400}/min^2.

in a separate tube since the presumed activator has esterolytic activity. As can be seen in Figure 3, the true V_∞ point fits on the line drawn through the other data points. Since the plasminogen to streptokinase mole ratio in these experiments are between 10 and 1.5 the discrepancy in V_∞ would be sufficiently large to be observed if there were a rapid preliminary activator formation. Therefore, the results of the first-order kinetic studies indicate that only one reaction is being measured and that the reaction is catalytic rather than stoichiometric.

Initial Velocity Studies. Due to the controversy as to whether streptokinase is an enzyme and whether more than one mole of plasminogen can bind to each mole of streptokinase, an initial velocity study was performed. The data illustrated in Figure 4 demonstrate that plots of the reciprocal of velocity of formation of plasmin vs. reciprocal of streptokinase concentration are linear. The replot of the (intercepts)$^{-1}$ of Figure 4 vs. streptokinase concentration is linear and goes through zero. This indicates that the V_{max} is directly proportional to the streptokinase concentration. The K_m for plasminogen is 25 μg and is not affected by the streptokinase concentration. These data indicate that the streptokinase activation of plasminogen obeys saturation kinetics and that there is only one site for plasminogen on the streptokinase molecule. Alkjaersig et al. (1957) have previously reported linear double reciprocal plots for the activation of human plasminogen by trypsin, urokinase, and streptokinase. However, they used only one level of streptokinase and interpreted their data to indicate only that the reaction was enzymatic. When benzoylphenylalanylvalylarginyl-*p*-nitroanilide was used as the chromogenic plasmin substrate in place of Cbz-lysine-*p*-nitrophyl ester linear double reciprocal plots were also obtained with similar values for K_m.

The possibility exists that there is a pre-steady-state formation of an activator complex which is too rapid to be measured and a slower reaction of the complex with a second molecule of plasminogen which would give linear double reciprocal plots. This reasoning is valid providing the plasminogen to streptokinase ratio is very large. Since the presumed streptokinase–plasminogen complex should have esterolytic activity (McClintock and Bell, 1971; Reddy and Markus, 1972; Schick and Castellino, 1974) and the mole ratio of plasminogen to streptokinase is as low as 5 in Figure 4, the pre-steady-state formation of the complex should give a significant burst of esterolytic activity. Since no burst of esterolytic activity has been observed (see Figure 1 where the mole ratio is about 10) activator formation is not occurring in these experiments.

Reddy and Markus (1972) reported that if streptokinase and plasminogen were incubated together the resulting streptokinase–plasmin complex was more active in converting plasminogen to plasmin than streptokinase alone. These workers measured plasminogen activation at only one time point (15 sec) and at least at the higher streptokinase concentrations were not measuring initial rates. Therefore, we performed the experiment illustrated in Figure 5. Streptokinase was incubated either alone or with plasminogen for 20 min. Under these conditions all the plasminogen is converted to plasmin. The resulting streptokinase or streptokinase–plasmin complex was then tested for its ability to activate plasminogen. Our results are in opposition to those of Reddy and Markus (1972) in that there is little difference between the two species and streptokinase alone is a slightly better activator than the streptokinase–plasmin complex. This difference may be due to degradation of streptokinase during the first incubation (Brockway and Castellino, 1974).

ε-Aminocaproic Acid Inhibition. When 2.2 μg of streptokinase and 44 μg of plasminogen are incubated at pH 7.0, complete conversion to plasmin is obtained by 20 min. At concentrations of ε-aminocaproic acid below 0.1 *M*, no inhibition of ester hydrolysis by plasmin is detectable. These results are in agreement with the data of Brockway and Castellino (1970) and those of Iwamoto et al. (1968) who reported that ε-aminocaproic acid is an excellent inhibitor of plasmin at concentrations above 0.1 *M*.

The activation of plasminogen to plasmin is inhibited by ε-aminocaproic acid at levels of 0.001 *M* (Iwamoto et al., 1968), or 0.2 *M* (Brockway and Castellino, 1970) and 0.1 *M* (Alkjaersig et al., 1959). Furthermore, the inhibition is reported to be competitive with respect to streptokinase concentration (Brockway and Castellino, 1970) and competitive with respect to plasminogen concentration in the reaction catalyzed by either urokinase or streptokinase

(Alkjaersig et al., 1959; Iwamoto et al., 1968). We have been able to obtain inhibition of the streptokinase activation of plasminogen at much lower concentrations of ϵ-aminocaproic acid. The inhibition is uncompetitive with respect to plasminogen (Figure 6) and the K_i is 0.6 mM as determined from the linear replot of the intercepts of Figure 6 vs. ϵ-aminocaproic acid concentration. It is not difficult to reconcile our results with others who have reported that ϵ-aminocaproic acid is a competitive inhibitor of plasmin formation. It should be noted that we are measuring initial rates of plasmin formation while Iwamoto et al. (1968) measured plasmin formation at a single time point which was well beyond the time where plasmin formation was linear with time. Alkjaersig et al. (1959) used levels of ϵ-aminocaproic acid which are inhibitory for plasmin activity as well as plasmin formation. Brockway and Castellino (1970) measured inhibition at varying levels of streptokinase and constant plasminogen at one time point of 10 min. In the absence of inhibitor and at similar levels of streptokinase their data indicated that all the plasminogen was converted to plasmin by 10 min. The observation of Brockway and Castellino (1970) that ϵ-aminocaproic acid inhibition is competitive with respect to streptokinase does not conflict with the view that streptokinase is a catalyst since any reversible inhibitor would be expected to be competitive with respect to the catalyst. Thus previous reports that ϵ-aminocaproic acid is a competitive inhibitor of plasminogen activation measured the inhibition vs. enzyme rather than vs. substrate, measured the reaction after significant amounts of substrate had been converted to the product, or utilized inhibitor levels that interfere with the assay of the product.

Discussion

Among the mechanisms which have been suggested for the activation of human plasminogen by streptokinase are (a) direct activation (Kline and Ts'so, 1971; Summaria et al., 1969), (b) prior formation of a streptokinase–plasmin or streptokinase–plasminogen complex which acts as a plasminogen activator (Kline and Fishman, 1961; Reddy and Markus, 1972, 1973, 1974; McClintock et al., 1974; Brockway and Castellino, 1974), and (c) interaction of streptokinase with plasmin, which contaminates human plasminogen, to form a modified streptokinase which can activate human plasminogen directly (Taylor and Beisswanger, 1973).

Mechanism c may be rejected on the basis of two lines of evidence: Brockway and Castellino (1974) demonstrated that plasmin-modified streptokinase and native streptokinase behave in an indistinguishable manner in plasminogen activation assays. Furthermore, streptokinase has been shown to be capable of activating human plasminogen after all the contaminating plasmin has been inactivated by either *p*-nitrophenyl-*p'*-guanidinobenzoate (McClintock and Bell, 1971; Reddy and Markus, 1972), diisopropyl phosphofluoridate, or 1-chloro-3-tosylamino-7-amino-2-heptanone (Summaria et al., 1969).

Our results provide an experimental test of mechanisms a and b. If streptokinase forms a complex with plasmin or plasminogen which in turn activates a second molecule of plasminogen, one would expect cooperative kinetics and curved double reciprocal plots. The linear double reciprocal plots shown in Figure 4 indicate that the activation of human plasminogen does not exhibit cooperative kinetics. However, it may be argued that if there were a rapid pre-steady-state formation of an activator complex, the reaction

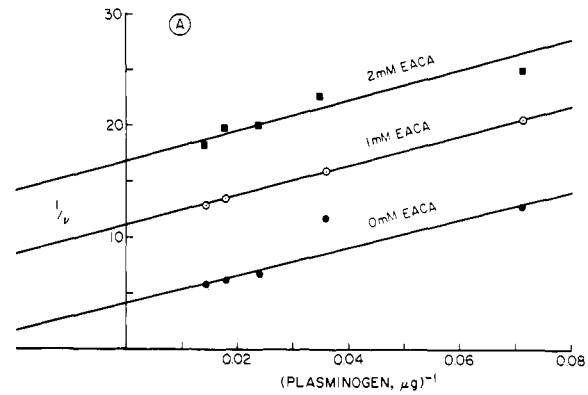

FIGURE 6: ϵ-Aminocaproic acid (EACA) inhibition of plasmin formation. Conditions were the same as in Figure 4 except streptokinase concentration was 1.68 μg/ml. ϵ-Aminocaproic acid concentration was 0 (●), 1 mM (○) or 2 mM (■). Velocity units are A_{400}/min^2.

may be written:

$$\mathrm{SK} + \mathrm{Plg} \overset{K_1}{\rightleftharpoons} \mathrm{SK{\cdot}Plg} \overset{K_2\,\mathrm{Plg}}{\rightleftharpoons} \mathrm{SK{\cdot}Plg{\cdot}Plg}$$

$$\mathrm{SK{\cdot}Plg{\cdot}Plg} \xrightarrow{k} \mathrm{SK{\cdot}Plg} + \mathrm{Pl} \overset{K_3}{\rightleftharpoons} \mathrm{SK{\cdot}Plg{\cdot}Pl}$$

where SK is streptokinase, Plg is plasminogen, and Pl is plasmin. The rate equation for this reaction is:

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_1K_2}{[\mathrm{Plg}^2]} + \frac{K_2}{[\mathrm{Plg}]} + \frac{K_2[\mathrm{Pl}]}{K_3[\mathrm{Plg}]}\right)$$

Since K_1 (the dissociation constant of the SK·Plg complex) is small, the $K_1K_2/[\mathrm{Plg}^2]$ term will be insignificant and double reciprocal plots will be linear. This reasoning is valid providing the Plg/SK ratio is very large. Since the presumed SK·Plg complex should have esterolytic activity (McClintock and Bell, 1971; Reddy and Markus, 1972; Schick and Castellino, 1974) and since the mole ratio of Plg/SK is as low as 5 in Figure 4, the initial formation of the complex should give a significant burst of esterolytic activity. Since no burst of esterolytic activity has been observed in these experiments (Figure 1) these data are not consistent with the formation of an activator complex. Furthermore, when streptokinase is incubated with an excess of plasminogen, so as to saturate the streptokinase with either plasmin or plasminogen, a plasminogen activator which has greater activity than streptokinase alone is not formed (Figure 5). The linear first-order plots in Figure 3 indicate that only one reaction is being measured and that this reaction is the formation of plasmin and not the second-order formation of the plasminogen activator (a 1:1 complex of streptokinase and plasminogen) since the pseudo-first-order rate constant is not affected by the plasminogen concentration (Tables I and II). If there were activator complex formation the first-order plots shown in Figure 3 would be nonlinear since the plasminogen to streptokinase mole ratios in these experiments are between 10 and 1.5. Thus, the activator complex would significantly contribute to the V_∞. Even if complex formation were too rapid to be measured, the lines would intersect the axis below the measured V_∞. Since the V_∞ point is on the line, only one reaction is being measured and that reaction can only be plasmin formation because the pseudo-first-order rate constant is proportional to the SK concentration and not dependent on the Plg concentration (Tables I and II). Also in these experiments more moles of plasminogen are utilized than moles of streptoki-

nase are present. Furthermore, neither the K_m nor the V_{max} for Cbz-lysine-*p*-nitrophenyl ester of the esterolytic activity generated in 20 min was altered by varying the mole ratios of streptokinase to plasminogen from 1:1 to 1:60 (Figure 2). If varying ratios of "plasminogen activator" to plasmin were generated in these mixtures, one would have expected to observe alterations in the K_m, the V_{max}, or both.

The streptokinase activation of human plasminogen differs from typical enzymatic reactions in that streptokinase has a very low turnover number and in most investigations, including our own, the mole ratio of plasminogen to streptokinase does not exceed 200. The low turnover may be due to the slow dissociation of the plasmin-streptokinase complex which is readily isolated from mixtures of the two compounds (Davies et al., 1964; DeRenzo et al., 1967b).

When streptokinase and plasminogen form a stoichiometric complex an active esterolytic site is formed before the formation of plasmin and this site has been shown by titration with [^{14}C]-*p*-nitrophenyl-*p'*-(amidinothiomethyl)-benzoate to be in the plasminogen moiety of the complex (Schick and Castellino, 1974). We propose that this active center can cleave the arginyl-valyl bond of the plasminogen portion of the complex to form a plasmin complex which then dissociates to free plasmin and streptokinase. There is, therefore, no need to postulate reaction of this complex with another plasminogen molecule. The proposed mechanism would predict that the uncompetitive inhibition observed with ϵ-aminocaproic acid is due to reaction with the transient complex, preventing cleavage of the arginyl-valyl bond and the subsequent formation of plasmin. If the alternate model were valid one would predict competitive inhibition for reaction of the inhibitor with the active site of the activator complex and noncompetitive inhibition if the ϵ-aminocaproate reacted with both the activator and ternary streptokinase-plasminogen-plasminogen complexes. In the proposed mechanism, streptokinase is functioning as a catalytic allosteric effector. The ability of streptokinase to increase the pseudo-first-order rate constant (Tables I and II) is consistent with its role as a catalyst.

Since it is well known that bovine plasminogen cannot be activated by streptokinase in the absence of human plasminogen, it is imperative that our proposed mechanism not be in conflict with this observation. A unified mechanism which is not in conflict with the idea of a direct activation of human plasminogen and a two-step process for the activation of bovine plasminogen is the following scheme where SK is streptokinase, HPlg is human plasminogen, HPl is human plasmin, BPlg is bovine plasminogen, and BPl is bovine plasmin:

$$\begin{array}{ccccccc} \text{SK} + \text{HPlg} & \rightleftharpoons & \text{SK·HPlg} & \longrightarrow & \text{SK·HPl} & \rightleftharpoons & \text{SK} + \text{HPl} \\ & & & & \Big\downarrow \text{BPlg} & & \\ \text{BPl} + \text{SK·HPl} & \rightleftharpoons & \text{SK·HPl·BPl} & \longleftarrow & \text{SK·HPl·BPlg} & & \end{array}$$

When human plasminogen is in excess, the dissociation of the SK·HPl complex is favored by competition for the streptokinase by the excess human plasminogen. Since bovine plasminogen has a low affinity for streptokinase (a streptokinase-bovine plasminogen complex has never been isolated) it cannot favor dissociation of the SK·HPl complex, in fact by being a substrate for the complex it will favor complex formation. If the bovine plasminogen also reacts with the SK·HPlg complex as well as the SK·HPl complex the above reasoning still holds except the bovine plasminogen will also slow the conversion of SK·HPlg to SK·HPl. It is possible that under conditions which would prevent dissociation of the SK·HPl, this complex could activate human plasminogen as well as bovine plasminogen. However, our results demonstrate that under our conditions, the complex is capable of dissociation. Thus, the above scheme allows both the direct activation of human plasminogen and the two-step activation of bovine plasminogen and is compatible with previously published observations.

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Purification and Properties of an Anti-B Hemagglutinin Produced by *Streptomyces* sp.†

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ABSTRACT: An anti-B hemagglutinin was purified to homogeneity from the culture filtrate of a strain of *Streptomyces* sp. by affinity chromatography. The *Streptomyces* hemagglutinin was adsorbed to insolubilized gum arabic and eluted with 1 *M* NaCl containing 1 *M* D-galactose. The purified hemagglutinin is thought to be homogeneous judging from sodium dodecyl sulfate–polyacrylamide gel electrophoresis at pH 7.2, disc gel electrophoresis at pH 4.3, isoelectric focusing, and ultracentrifugation. The molecular weight was estimated to be 11,000 from results of gel filtration in 6 *M* guanidine hydrochloride (Gdn·HCl), sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and sedimentation equilibrium analysis. The amino acid analyses revealed that the hemagglutinin contained large amounts of alanine, glycine, and valine, 47% of the total amino acid residues, and no phenylalanine. Carbohydrate analysis demonstrated that the hemagglutinin might not be a glycoprotein. The circular dichroic (CD) spectrum of the protein is quite different from those of usual proteins in having a large positive peak at 226 nm (θ = 10,000) and a negative band at 212 nm (θ = −2600). The hemagglutinin showed a typical precipitation curve with gum arabic, and agglutinated human blood group B erythrocytes 256 times as strongly as A or O erythrocytes. These activities were not affected by pH (from 4 to 12). The anti-B activity was further confirmed by serological tests. The hemagglutination-inhibition studies indicated that D-galactose was inhibitory, but α-D-galactosides were not necessarily better inhibitors than β-D-galactosides. L-Rhamnose was the best inhibitor among the monosaccharides tested, and L-arabinose and D-fucose were also inhibitory.

Since blood group specific hemagglutinins were found in the extracts of certain plant seeds by Renkonen (1948) and Boyd and Regura (1949), numerous agglutinins have been purified to homogeneity from plants and animals (Sharon and Lis, 1972; Oppenheim et al., 1974). Many anti-A and some anti-H hemagglutinins occur in plants; however, hemagglutinins with anti-B activity had not been found until the *Streptomyces* agglutinin was reported in 1973 (Fujita et al., 1973). A lack of anti-B hemagglutinins has restricted the use of hemagglutinins as serological reagents.

Recently, Hayes and Goldstein (1974) purified and characterized an anti-B hemagglutinin from extracts of *Bandeiraea simplicifolia* seeds. Though plant agglutinins generally bind to *N*-acetyl-D-galactosamine more strongly than D-galactose (Etzler and Kabat, 1970; Galbraith and Goldstein, 1972; Poretz et al., 1974), the *B. simplicifolia* agglutinin binds to D-galactose 15 times more strongly than *N*-acetyl-D-galactosamine. The *Streptomyces* agglutinin specifically agglutinates blood-group B erthrocytes and appears to bind to D-galactose 60 times more strongly than *N*-acetyl-D-galactosamine, based on the results of hemagglutination-inhibition tests with a partially purified sample. Thus, the *Streptomyces* agglutinin is probably more specific to blood group B erythrocytes than *B. simplicifolia* agglutinin and will be very useful as a serological reagent.

This report describes the purification of the *Streptomyces* hemagglutinin and characterization of the purified hemagglutinin concerning its purity, specificity, and some of its physicochemical properties.

Materials and Methods

Cultivation. Streptomyces 27S5 was kindly supplied by Professor H. Yonehara, Institute of Applied Microbiology, University of Tokyo. The bacteria were grown in a 30-l. jar fermenter under the same conditions as described previously (Fujita et al., 1973). Each jar containing 15 l. of medium was inoculated with 400 ml of a log phase culture. Growth was continued for 4 days at 27°. At the end of this period, the hemagglutinating activity reached a plateau of about 2 hemagglutination titer, and the culture broth was harvested by filtration.

Assays. Assays of hemagglutinating activity were performed with a microtiter apparatus (Cooke Engineering Co., Alexandria, Va.) using a 2% human A, B, O, or AB erythrocyte suspension and 0.15 *M* NaCl as diluent. Hemagglutination was conducted for 90 min at room temperature. The activity was expressed as titer, the reciprocal of the highest twofold dilution exhibiting positive hemagglutination. For inhibition studies, purified hemagglutinin (titer,

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