Mechanism of Formation of Bovine Plasminogen Activator from Human Plasmin*

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It has been shown that the activation of bovine plasminogen by streptokinase plus a trace amount of human globulin involves a two-stage activation process (1, 2). Ablondi and Hagan (3) proposed that the proactivator of bovine plasminogen was identical with human plasminogen, which became activator by forming complexes with streptokinase. Kline and Fishman (4) using a lysine methyl ester assay demonstrated that the kinetics of inactivation of plasmin was similar to that of proactivator. They stated that plasminogen activator might be formed from plasmin plus streptokinase. The same suggestion was made by Zylber, Blatt, and Jensen (5) and Blatt, Gray, and Jensen (6). However, all the available data did not rule out the possibility that the activator could exist in the form of a plasmin-streptokinase complex. Recently, Markus and Werkeheiser (7, 8) postulated the existence of two proactivators in human blood; one (proactivator I) supposedly activated both human and bovine plasminogen while the other (proactivator II) activated bovine plasminogen only. It has also been suggested that both human plasmin and plasminogen could serve as proactivator and form activator with streptokinase (7-9).

In the present communication, methods and experiments are presented to elucidate the mechanism of formation of bovine plasminogen activator from human plasmin. It will be shown that human plasmin and not human plasminogen is the immediate precursor of bovine plasminogen activator and is, therefore, the proactivator.

Materials and Methods

Native Human Plasminogen and Human Plasmin—Native human plasminogen and human plasmin were prepared from plasma Fraction II2,3 by the method described by Robbins (11) followed by chromatography on DEAE-Sephadex and gel filtration on Sephadex (12).

Acid-treated Human Plasminogen and Human Plasmin—Acid-treated human plasminogen and human plasmin were prepared from plasma Fraction II2,3 by a modification of the Kline method (11) followed by chromatography on DEAE-Sephadex and gel filtration on Sephadex (12).

Bovine Plasminogen and Bovine Plasmin—Bovine plasminogen and bovine plasmin were obtained from Parke, Davis and Company. The specific activities of these preparations as determined by the modified Remmert and Cohen casein assay (12) (see below) were 0.4 unit per mg for bovine plasminogen and 0.2 unit per mg for bovine plasmin.

Streptokinase—The streptokinase used was a commercial preparation, Varidase (Lederle Laboratories). A preparation of high purity streptokinase with a specific activity of 90,000 units per mg of protein was kindly supplied by Dr. E. C. De Renzo, Lederle Laboratories.

c-Aminocaproic Acid—c-Aminocaproic acid was a product of Mann Research Laboratories.

Results

Human Plasminogen or Human Plasmin as Proactivator—In the absence of an inhibitor, both human plasmin and human plasminogen could serve as proactivator with which streptokinase formed activator of bovine plasminogen. The activator activity of streptokinase-human plasmin mixtures was tested with varying amounts of human plasmin against a constant quantity of streptokinase. It was found that the activator activity reached a maximum at a streptokinase-human plasmin ratio of about 750 (Fig. 1A). When the amount of human plasmin or human plasminogen was constant, the activator activity was a function of the amount of streptokinase. A saturation of human plasmin or human plasminogen was shown when the streptokinase to human plasmin ratio was about 3000 (unit per unit) (Figs. 1B and 2). The activator activity of the human plasminogen or human plasmin (0.066 unit) used per assay was negligible.

Human Plasminogen or Human Plasmin as Proactivator—In the absence of an inhibitor, both human plasmin and human plasminogen could serve as proactivator with which streptokinase formed activator of bovine plasminogen. The activator activity of streptokinase-human plasmin mixtures was tested with varying amounts of human plasmin against a constant quantity of streptokinase. It was found that the activator activity reached a maximum at a streptokinase-human plasmin ratio of about 750 (Fig. 1A). When the amount of human plasmin or human plasminogen was constant, the activator activity was a function of the amount of streptokinase. A saturation of human plasmin or human plasminogen was shown when the streptokinase to human plasmin ratio was about 3000 (unit per unit) (Figs. 1B and 2). All the streptokinase-human
plasmin or human plasminogen ratios described refer to unit to unit ratios, unless specified otherwise. At higher streptokinase levels, the activator activity of streptokinase-human plasmin mixtures remained almost constant while the activator activity of streptokinase-human plasminogen mixtures declined markedly with increasing streptokinase levels (Fig. 2).

**Calculation of Streptokinase to Human Plasmin Ratio for Activator Formation**—When the amount of streptokinase was kept constant and the amount of human plasmin was varied, bovine plasminogen activator increased linearly with increasing amounts of human plasmin until the maximum activator activity was approached. Similarly, when the amount of human plasmin was kept constant and the amount of streptokinase was varied, a similar curve was obtained with respect to activator activity and to the amount of streptokinase used (Fig. 1). Assuming that the percentage of activator activity appearing in either experiment was a measure of the percentage of the constant component that had been converted to activator, the equivalent amounts of streptokinase and human plasmin needed to form activator would be the amount of either constant component (calculated from the percentage of activator activity and the total amount of either constant component) that gave the same activator activity. For example, 50% activator activity in Fig. 1A represents 50% of the 50 units of streptokinase (or 25 units) that reacted to form activator giving an absorbance increase equal to 0.06. The same extent of absorbance increase in Fig. 1B was given by 32.5 units of total human plasmin or 0.011 unit. Then, 25 units of streptokinase should have reacted with 0.011 unit of human plasmin to form activator. Assuming a specific activity of 90,000 units per mg of protein and a molecular weight of 47,000 (15) for the high purity streptokinase, and a specific activity of 24 units per mg of protein and a molecular weight of 89,000 (10) for the highly purified plasmin, then the above streptokinase to human plasmin ratio was calculated to be 1.1 on a molar basis.

**Streptokinase Effect on Proteolytic Activity of Human Plasmin and Bovine Plasmin**—The effect of streptokinase on the proteolytic activity of bovine plasmin, human plasmin, and human plasminogen was studied with varying amounts of streptokinase (Fig. 3). The bovine plasmin activity was not affected up to a streptokinase to bovine plasmin ratio of 30,000, whereas human plasmin activity began to decline sharply when the streptokinase to human plasmin or streptokinase to human plasminogen ratio was increased from 600 to 30,000. However, when the results shown in Fig. 3 are compared with the results shown in Fig. 2, it can be seen that the increasing activator activity obtained with increasing streptokinase to human plasmin or streptokinase to human plasminogen ratios was not related to the decline of proteolytic activity in the mixture. When the ratio was 600, the proteolytic activity of human plasmin (or the activated human plasminogen) was still at its maximum but the activator activity had already reached 50% of its maximum. On the other hand, at a ratio of about 6,000, the activator activity had reached its maximum but the proteolytic activity had only decreased to about 40% of maximum. The proteolytic activity disappeared completely at ratios of about 30,000.

**Kinetics of Bovine Plasminogen Activator Formation**—When either human plasminogen or human plasmin was first incubated with high ratios of streptokinase at 25°C for 5 min, before mixing with bovine plasminogen, both streptokinase-human plasminogen and streptokinase-human plasmin mixtures showed maximal activator activity in activating bovine plasminogen. However,
if human plasminogen was first added to bovine plasminogen before the addition of streptokinase, the activation of bovine plasminogen was very limited (less than 30% of maximal activation). The formation of activator from human plasminogen and streptokinase was inhibited by bovine plasminogen. This finding permitted us to study the kinetics of bovine plasminogen activator formation. Human plasmin or plasminogen, in an amount of 0.066 casein unit, was mixed with 200 units of streptokinase in 0.5 ml of 0.067 M potassium phosphate buffer, pH 7.4, and incubated at 25°. At various time intervals, one of the mixtures was withdrawn and frozen in a Dry Ice-ethanol bath. Bovine plasminogen in an amount of 2.5 casein units in 0.5 ml of 0.067 M potassium phosphate buffer, pH 7.4, was added to each of the frozen mixtures, and the final mixtures were then incubated at 25° for 10 min. The proteolytic activity of the activated bovine plasminogen was then determined in the casein assay. It was found that while complete activation of the proactivator in the streptokinase-human plasmin mixture was almost instantaneous, it took 2 min for the streptokinase-human plasminogen mixture to acquire maximal activator activity.

Effect of $\varepsilon$-Aminocaproic Acid on Formation of Bovine Plasminogen—$\varepsilon$-Aminocaproic acid has been found to be a potent inhibitor of bovine plasminogen and human plasminogen activation (16, 17). The formation of $\varepsilon$-aminocaproic acid on the formation of bovine plasminogen activator was studied in the following experiments. Human plasminogen (2 units) was mixed with various amounts of streptokinase in 1.5 ml of 0.067 M potassium phosphate buffer, pH 7.4, containing 0.1 M $\varepsilon$-aminocaproic acid and incubated at 25° for 5 min. Then, 0.05 ml of each of the mixtures was added to 2.5 units of bovine plasminogen in 1.0 ml of the same buffer (the final $\varepsilon$-aminocaproic acid concentration was 0.005 M). The final mixture was incubated at 25° for 10 min before assaying for proteolytic activity. The same procedure was repeated with human plasmin in the place of human plasminogen. As shown in Fig. 4, the activation of bovine plasminogen by the streptokinase-human plasminogen-$\varepsilon$-aminocaproic acid mixture was almost negligible, while the activation by the streptokinase-human plasmin-$\varepsilon$-aminocaproic acid mixture was about 50% of the control (no $\varepsilon$-aminocaproic acid).

Another experiment was designed to test whether the 50% inhibition obtained with the human plasmin mixture was due to $\varepsilon$-aminocaproic acid inhibition of the step in which bovine plasminogen was activated by the activator. The experiment was essentially the same as that described above, except that the streptokinase to human plasmin or streptokinase to human plasminogen ratios were constant at 3000, and various amounts of $\varepsilon$-aminocaproic acid were added to the human plasmin or human plasminogen solutions before the addition of streptokinase or after the 5-min incubation period of the streptokinase-human plasmin or streptokinase-human plasminogen mixtures (for activator formation). The results of this experiment showed that in all cases the proteolytic activity (bovine plasmin) formed in the subsequent 10-min period (for bovine plasminogen activation) decreased as the inhibitor concentration increased (Fig. 5). However, if inhibitor was present during the first 5-min activator formation period, the streptokinase-human plasmin mixture gave much less activator activity than the streptokinase-human plasmin mixture. If inhibitor was added to the activator mixtures after the activator formation period, both the streptokinase-human plasminogen and streptokinase-human plasmin mixtures were identical in activator activity, which was also equal to that of the streptokinase-human plasmin mixture to which $\varepsilon$-aminocaproic acid was added before the mixing of human plasmin and streptokinase. The same results were obtained with either Varidase or high purity streptokinase.

The $\varepsilon$-aminocaproic acid inhibition of bovine plasmin activity was determined in order to see how much inhibition was contributed by the amount of inhibitor used in the activator experiments (Fig. 6). The highest inhibitor concentration in the final
Fig. 5. e-Aminocaproic acid inhibition of activator formation at various inhibitor concentrations. A, inhibitor concentrations during activator formation; B, inhibitor concentrations during bovine plasminogen activation. Inhibitor present during activator formation, human plasminogen (△—△) or human plasmin (○—○); inhibitor added after activator formation, human plasminogen (△—△) or human plasmin (○—○). See text for details.

Fig. 6. e-Aminocaproic acid inhibition of bovine plasmin assay mixture was 0.0012 M. This concentration of e-aminocaproic acid gave about 5% inhibition of the proteolytic activity of bovine plasmin, which was rather insignificant.

The above experiments, with a “differential e-aminocaproic acid inhibition technique” to study the bovine plasminogen proactivator activity of human plasmin and human plasminogen preparations, have also been extended to the acid-treated human plasminogen and plasmin preparations. As far as bovine plasminogen proactivator activity was concerned, the acid-treated preparations behaved very much the same as the native enzyme (proenzyme).

DISCUSSION

e-Aminocaproic acid has been known to be a potent inhibitor for the activation of both bovine plasminogen and human plasminogen (16, 17). It has been discouraging that a concentration of e-aminocaproic acid low enough to allow the activation of bovine plasminogen did not efficiently inhibit the conversion of human plasminogen to plasmin. On the other hand, a high e-aminocaproic acid level sufficient to prevent the latter conversion would also inhibit bovine plasminogen activation even if activator was preformed. In our studies, an experimental procedure was designed in such a way that high e-aminocaproic acid concentrations were used during the course of activator formation from either human plasmin or human plasminogen and streptokinase, but, by a dilution procedure, much lower inhibitor concentrations were present during the time of activation of bovine plasminogen. It was found that 0.1 M e-aminocaproic acid almost completely prevented the formation of activator from human plasminogen and streptokinase but did not inhibit the formation of activator from human plasmin and streptokinase. This concentration of e-aminocaproic acid was present only during the time of formation of activator from its precursor (the first 5-min preincubation period). A 20-fold dilution of this concentration of inhibitor was used in the course of bovine plasminogen activation (the subsequent 10-min preincubation period), and a 80-fold dilution was used in the final assay. This procedure allowed us to study the differential inhibition of the transformation of human plasminogen to human plasmin and the formation of activator. The data presented in Fig. 5 showed that, as far as the formation of activator is concerned, once human plasminogen had been activated to human plasmin, e-aminocaproic acid was no longer inhibitory. Although human plasmin which served as the proactivator was also slightly inhibited by e-aminocaproic acid, the inhibition was not on the formation of activator but on the activation of bovine plasminogen by the activator because the same results were obtained when e-aminocaproic acid was added before or after the formation of activator (the first 5-min preincubation). In these experiments, advantage was also taken of the fact that the addition of bovine plasminogen to human plasminogen prior to the addition of streptokinase largely prevented the formation of the activator from human plasminogen and streptokinase. Otherwise, activator formation would have occurred and continued to completion after the dilution of e-aminocaproic acid. These findings were in agreement with Kline’s report that e-aminocaproic acid at a concentration of 0.025 M had no effect on activator production from human plasmin and streptokinase (18).

Based on the above findings, the reaction sequence of bovine plasminogen activation may be shown.

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\begin{align*}
\text{Human plasminogen} & \xrightarrow{\text{streptokinase}} \text{human plasmin (proactivator)} \\
\text{Human plasmin + streptokinase} & \rightarrow \text{human plasmin-streptokinase (activator)} \\
\text{Bovine plasminogen} & \xrightarrow{\text{human plasmin-streptokinase}} \text{bovine plasmin}
\end{align*}
\]

As shown above, Reactions 1 and 3 were inhibited by e-aminocaproic acid. Reaction 1 is only a phenomenal description rather than an actual chemical sequence. Markus and Werkheiser (7, 8) presented an interesting concept and experimental data showing that human plasminogen was activated by a
plasminogen-streptokinase to human plasmin-streptokinase and De Renzo (15) demonstrated by ultracentrifugal studies totally impossible. If this is true, the conversion of human streptokinase (activator) through this alternative pathway is not of our findings. However, the formation of human plasmin-bovine plasminogen activator does not seem to be likely in view of the reactions which might also occur. The possibility that human plasmin-streptokinase is a bovine plasminogen activator does not seem to be likely in view of our findings. However, the formation of human plasmin-streptokinase (activator) through this alternative pathway is not totally impossible. If this is true, the conversion of human plasmin-streptokinase to human plasmin-streptokinase must also be inhibited by e-aminocaproic acid. Davies, Englert, and De Renzo (15) demonstrated by ultracentrifugal studies the formation of a complex equivalent to a 1:1 (mole per mole) combination of human plasminogen and streptokinase in the presence of e-aminocaproic acid. The authors pointed out that it was possible that such a complex was actually a human plasmin-streptokinase complex. The latter postulate does not seem to be tenable because of the use of e-aminocaproic acid in their system.

Zylber, Blatt, and Jensen (5) and Blatt, Segal, and Gray (19) reported the existence of a plasminogen activator which was a mole per mole complex of human plasmin and streptokinase. The formation of this human plasmin-streptokinase complex or the human plasminogen-streptokinase complex of Davies, Englert, and De Renzo (15) appeared to involve, as pointed out by the latter authors, “either an irreversible process, or that, if an equilibrium exists, the formation of the complex is greatly favored.” However, our experimental data revealed that when the amount of human plasminogen or human plasmin was kept constant and that of streptokinase varied, the maximum activator activity appeared at streptokinase to human plasmin or human plasminogen ratios (unit per unit) of about 3:000. But, when the amount of streptokinase was constant and that of human plasmin varied, a streptokinase to human plasmin ratio (unit per unit) of about 750 gave the maximum activator activity. Assuming the specific activity of “pure” streptokinase to be 90,000 units per mg of protein, and since the specific activity of highly purified human plasminogen prepared in our laboratory was approximately 24 casein units per mg of protein, a mole per mole ratio of streptokinase to human plasmin or human plasminogen giving the maximal activator activity described above should be 1:0.7 and 1:2.6, respectively, based on molecular weights of 89,000 for human plasminogen and plasmin and a molecular weight of 47,000 for streptokinase. Since the maximal activator activity appeared at different streptokinase ratios when the amount of either streptokinase or human plasmin was kept constant and the other was a variable, it tends to indicate that in order to saturate one component, the other component has to be in excess and that the formation of the activator from its precursors involves a reversible process as suggested by Mullertz (20). It is interesting to note that the molar ratio of streptokinase and human plasmin calculated from the activator activity generated from both components (Fig. 1) happened to be close to unity (1.1).

The inhibition of proteolytic activity of human plasmin by high levels of streptokinase has been previously reported by other workers (7, 19, 21). Blatt, Segal, and Gray (19) used the extent of streptokinase inhibition of proteolytic activity of human plasmin as a measure of complex formation. Markus and Werkheiser (7) further demonstrated that, with increasing streptokinase levels in a human plasminogen solution “activator II” activity rose at the expense of the human plasmin activity with azocasein as the substrate. However, bovine plasmin activity was reported not to be affected by high levels of streptokinase (21). In our studies, these findings were confirmed to some extent. However, it must be pointed out that the streptokinase inhibition of proteolytic activity may not be a direct result of bovine plasminogen activator formation (Figs. 2 and 3).

At high streptokinase levels the formation of activator from human plasmin appeared to level off due to the saturation of the latter, whereas the formation from human plasminogen declined after a maximum was attained (Fig. 2). If the proposed pathway of activator formation, i.e. from human plasminogen to...
human plasmin and then to activator, is the only way of activator generation, it is probable that high levels of streptokinase inhibited the activation of human plasminogen to human plasmin and, therefore, the formation of activator.

**SUMMARY**

The bovine plasminogen proactivator activities of highly purified human plasminogen and human plasmin were studied in the presence of streptokinase with a caseinolytic assay. A method is described for the estimation, by kinetic data, of the ratio of streptokinase to human plasmin which forms the activator. This ratio was estimated to be 1. The effect of streptokinase on the proteolytic activities of human plasmin and bovine plasmin were studied. It was found that high levels of streptokinase inhibited human plasmin but had no effect on bovine plasmin. An experimental procedure with "a differential e-aminocaproic acid inhibition technique" was designed to study the identity of the proactivator. It was found that e-aminocaproic acid inhibited the formation of activator from human plasminogen-streptokinase mixtures, but not from human plasmin-streptokinase mixtures. This evidence supports the hypothesis that human plasmin and not human plasminogen is the immediate precursor of bovine plasminogen activator.

**REFERENCES**

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