Mechanism of Activation of Human Plasminogen by the Activator Complex, Streptokinase·Plasmin*

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The object of this investigation was to distinguish between two potential mechanisms of activation of human plasminogen (HPg) to plasmin (HPm) by catalytic levels of the activator complex, streptokinase·plasmin (SK*·HPm). One mechanism, which is widely supported, postulates an enzymatic role for SK*·HPm in the conversion of molar excesses of plasminogen to plasmin. A more recently described kinetic mechanism involves a direct conversion of HPg to HPm by streptokinase (SK). Here, it is believed that displacement of HPm from SK*·HPm by excess HPg is the major source of free HPm in the activation process. The present paper shows that SK* is not capable of undergoing rapid exchange from SK*·HPm to other HPg or HPm molecules, thus precluding the possibility of direct activation of HPg by SK. Our evidence supports a mechanism involving an enzymatic role for SK*·HPm as the major means of converting free HPg to HPm.

The activation of human plasminogen (HPg) by catalytic levels of SK has been a widely studied subject. Any activation mechanism proposed for HPg must account for the cleavage of at least two peptide bonds in the activation process (1–7). One peptide bond cleavage, which does not lead to HPm activity, has been clearly shown to result from plasminolysis, and it now appears that the major activator (14) of HPg is a complex of streptokinase·plasmin (SK*·HPm), consisting of a proteolytically modified streptokinase (SK*) (15–17). This activator complex arises from the following sequence of reactions (9, 15, 18).

$$SK + HPg \rightarrow SK \cdot HPg \rightarrow SK \cdot HPg^* \rightarrow SK \cdot HPm \rightarrow SK + HPm$$

Here, SK and HPg form an initial complex (SK·HPg) which then undergoes a transition (13, 15, 19), allowing formation of a complex, SK·HPg*, which possesses an active site in the modified plasminogen moiety (20). This active site intramolecularly (9, 21) converts HPg to HPm in the complex and ultimately yields SK*·HPm. The complex SK*·HPm can also arise from interaction of SK with free HPg (14, 15, 16). Although SK·HPg* can potentially activate free HPg to HPm (15, 18, 19), its very limited stability, under the usual conditions of activation, does not strongly implicate this complex as the major plasminogen activator. The complex SK*·HPm has been shown in several studies to be capable of activation of human plasminogen to plasmin (14, 15, 18, 22).

It has been widely accepted that SK*·HPm acts in an enzymatic role in conversion of free Pg to Pm in any species. Inactivation of the plasmin-active site in this complex by agents such as diisopropylfluorophosphate (23, 24) has led to loss of activity of this complex in plasminogen activation. However, it has been recently proposed (21), based on kinetic data, that SK can directly activate HPg in a series of exchange reactions. Here, the following sequence of reactions has been proposed:

$$SK \cdot HPm + HPg = SK \cdot HPg + HPm$$

Basically, excess HPg displaces HPm from SK*·HPm, leading to formation of free HPm. Additional SK*·HPm is formed from SK*·HPg by the series of reactions described in sequence 1 and again displaced by HPg. We have previously shown, in a different context, that isolated SK* is capable of substituting for native SK in reaction sequence 1 above (18). This is a critical finding in order for the mechanism proposed by Kosow (21) to have credibility.

This latter plausible mechanism (sequence 2 above) has led to stimulating thoughts concerning the activation of HPg to HPm by catalytic levels of SK. In this paper, we describe several experiments which we believe have enabled us to determine the importance of mechanism 2 in the activation process.
Experimental Procedures

Preparation of Proteins—HPg was prepared from Cohn Fraction III (Cutter Laboratories) by affinity chromatography on Sepharose-lysine (26). As previously described (26). In most experiments, HPg affinity chromatography form 2 (26) was utilized. Rabbit plasminogen was prepared from whole citrated rabbit plasma in a similar fashion (5).

Human plasminogen was lightly iodinated with Na<sup>125</sup>I, according to the solid-state lactoperoxidase method (27). A quantity of 12 mg of HPg form 2 was dissolved in 2 ml of 0.1 M sodium phosphate buffer, pH 7.0. This was added to 0.5 ml of Sepharose-lactoperoxidase, prepared as previously described (28). Next, 0.05 ml of 10<sup>-3</sup> M KI and 0.05 ml of Na<sup>125</sup>I (0.05 mCi) were added. Iodination was initiated by the addition of 0.1 ml of 8% H<sub>2</sub>O<sub>2</sub>. This mixture was stirred for 30 min at room temperature. After centrifugation to remove the solution was extensively dialyzed against H<sub>2</sub>O until the radioactivity in the dialysate was negligible. The 125<sup>1</sup>-HPg obtained was fully activatable by SK and urokinase, at rates identical with that of native HPg. The resulting specific radioactivity of 125<sup>1</sup>-HPg was approximately 2 x 10<sup>6</sup> cpm mg<sup>-1</sup>, corresponding to approximately 0.2 to 0.4 mol of iodine/mg of HPg.

Basic pancreatic trypsin inhibitor (PTI) was purchased, under the trade name Trasylol, from FBA Pharmaceuticals in a high state of purity. The material was iodinated, with Na<sup>125</sup>I, as previously described (5). The specific radioactivity of 125<sup>1</sup>-PTI was in the range of 10<sup>6</sup> to 10<sup>7</sup> cpm/mg.

 Soybean trypsin inhibitor (STI) was purchased from Sigma and insolubilized on Sepharose 4B as described earlier (5). Streptokinase was obtained from Kabkin, obtained from ABKabi. Our methods for the purification of high quality SK have been published (29). The complexes SK 125<sup>1</sup>-HPg, SK<sup>+</sup> 125<sup>1</sup>-HPm, SK<sup>+</sup> 125<sup>1</sup>-PTI, SK·HPm·PTI, and SK·HPg·PTI were prepared and purified according to our published procedures (9).

Plasminogen Activator Assays—The plasminogen activator capability of SK<sup>+</sup>·HPm under different conditions was determined by analysis of the ability to convert rabbit Pg to Pm. This species of plasminogen is very weakly sensitive to activation by streptokinase alone under the conditions used here. Preformed activator, SK<sup>+</sup>·HPm, was prepared by incubating SK in equimolar quantities with HPg or HPm and incubating for 15 min at room temperature.

Since the activation experiments described in the manuscript varied under greatly different conditions, the exact incubation conditions are described in the text. The following general protocol was used to assay the amount of RPm formed. A given quantity of RPg was dissolved in a buffer consisting of 0.1 M sodium phosphate, 0.01 M lysine, pH 7.5. The activation of RPg was accelerated by addition of a given amount of the desired activator complex (e.g., SK<sup>+</sup>·HPm). At chosen intervals an aliquot of this solution was added to another solution, containing a 5-fold molar excess of PTI over the initial level of rabbit Pg. This solution was then passed over a small column (0.5 x 2.5 cm) of Sepharose-lysine, equilibrated with the incubation buffer. A K<sub>2</sub>CO<sub>3</sub> solution was used to rapidly force the solution over the column. Excess PTI was then washed from the column, again using pressure. The radiolabeled RPm, containing catalytically radiolabeled activator complex, as well as nonlabeled, unactivated (if any) RPg was eluted. The elution was carried out by addition of 1.5 ml of 0.1 M sodium phosphate, 0.1 M e-aminocaproic acid, pH 7.5, and collecting the eluate directly into a scintillation vial. This step was repeated with another 1.5-ml wash and collected in another scintillation vial. Control experiments indicated that virtually 100% of RPg, RPm, and SK·HPm was recovered by this procedure. In this manner a sharp amount of RPm present was readily quantitated. We have previously developed this technique to monitor the rate of active site formation in SK·HPg (30). By appropriate adjustment of the initial conditions, this technique is readily adapted for assessment of levels of Hp of SK<sup>+</sup>·HPm when desired.

Assays of the esterolytic activity of human or RPm or SK<sup>+</sup>·HPm were performed utilizing N<sup>-</sup>tosyl-L-arginine methyl ester as the substrate, and the potentiometric assay (31).
This resulting SK*·HPm complex was then used as a reference to monitor the rate of activation of RPg (22 nmol at a final volume of 0.3 ml). Rabbit plasminogen is weakly activated by SK alone and readily activated by the SK*·HPm complex. A similar experiment was performed as the above, except that the 0.46 nmol of SK was added to a solution which contained a mixture of 0.46 nmol of HPm and 0.46 nmol of HPm-PTI, and allowed to incubate for 5 to 20 min prior to addition of RPg. As can be seen from Fig. 2, the initial rate of activation of RPg with the latter activator solution was approximately 80% of that found with the reference activator solution. This result is independent of the preincubation time of SK with the activator solution, suggesting that a steady state was attained. If random binding of SK to HPm and HPm·PTI had occurred, then 50% of the rate of the reference solution should have been observed. In another experiment, 0.46 nmol of SK was added to a solution containing 0.46 nmol of HPm and 0.92 nmol of HPm·PTI. When this mixture was utilized to activate RPg, the initial velocity of activation corresponded to a quantity of 0.29 nmol of SK·HPm. Again, this is higher than theoretically predicted, assuming random binding of SK to HPm and HPm·PTI. These experiments indicate that significantly tighter binding of SK to HPm, compared to HPm·PTI, occurred. Thus, there is no artificial stabilization of SK to the inhibited activator complexes; and we feel that exchange of SK or SK* from the activator complex to uncomplexed HPg or HPm does not occur.

Final proof that there is a lack of rapid exchange of SK or SK* when bound to HPm comes from our studies reported below. We have prepared $^{125}$I·HPg as described under "Experimental Procedures." The material was completely activated to $^{125}$I·HPm by insolubilized urokinase (7) and purified by adsorption to Sepharose-lysine. An SK*·$^{125}$I·HPm activator complex was prepared by incubation of 0.02 nmol of $^{125}$I·HPm with 0.63 nmol of SK in a final volume of 0.015 ml for 5 min at room temperature. The buffer utilized for all experiments was 0.1 M sodium phosphate, 0.01 M lysine, pH 7.5. It was found that this complex did not bind to a column of Sepharose-2BTI, whereas, HPm was completely adsorbed to this column, in agreement with an earlier study (33). A quantity of 0.005 ml of the SK*·$^{125}$I·HPm (0.2 nmol) was mixed with 0.05 ml of HPg (3.25 nmol) and incubated for times of 15 min and 30 min. Separate studies showed that complete conversion of HPg to HPm occurred within 10 min. The mixture was then passed over a column (0.5 x 2.5 cm) of Sepharose-2BTI to completely adsorb the HPm generated. The radioactivity in the wash, identified as SK*·$^{125}$I·HPm, could only be generated by transfer of SK from SK·$^{125}$I·HPm to HPm·PTI. The methods have been described under "Experimental Procedures" and "Results."
DISCUSSION

The major goal of this work was to evaluate two potentially important mechanisms whereby the plasminogen activator complex SK*-HPm is able to serve as a catalyst for activation of any species of plasminogen to plasmin. It was widely believed that SK*-HPm served as an enzyme for activation of HPg. In this mechanism, the active site of HPm, which does not convert HPg to HPm, is modified by binding of SK, such that plasminogen activation is now capable of occurring (34). The chief physical evidence for this was that blockage of the plasmin-active site in the complex by agents such as diisopropylfluorophosphate (23, 24) or p-nitrophenyl-p'-guanidinobenzoate (15) led to complete loss of plasminogen activator ability of any activator complex.

More recently a kinetic mechanism for the activation of HPg by SK*-HPm has been described (21). Basically, in this mechanism it is believed that in the SK*-HPm complex, HPg will displace HPm and lead to free HPm and a complex of SK*-HPg. This latter complex is thought to recycle SK*-HPm by the series of reactions described in sequence (1) in the introduction. The recycled SK*-HPm again forms SK*-HPg and continues as previously. This exchange mechanism cannot be ruled out by the studies mentioned in the previous paragraph, in which complete loss of the activator ability of the plasminogen activator complex(es) was attained upon specific inhibition of the HPm-active site in the complex. The reason for this is that the binding of SK* to the inhibited HPm may be stronger than its binding to uninhibited HPm, thus, artificially precluding exchange. In fact, it has been shown (15) that SK binds with higher affinity to HPm which has been acylated by p-nitrophenyl-p'-guanidinobenzoate than to unacylated HPm. For the plasmin exchange mechanism to have merit, it must be shown that isolated SK*, when mixed with HPg, can indeed form SK*-HPm. In a different context, we have shown that SK* can serve in this manner (17, 28).

Due to the above considerations, we sought a HPm and SK*-HPm inhibitor which was rapid, specific, and did not lend enhanced affinity for SK* to HPm. All of the above criteria are satisfied by the inhibitor PTI. The data in Fig. 2 clearly demonstrate that HPm PTI, although capable of extremely tight binding of SK, is not as effective as HPm in this regard. Thus, the data represented in Fig. 1 can be interpreted to strongly indicate that SK or SK* exchange did not occur from SK*-HPm to HPg. This is confirmed by the results of Table I, which show that when SK*-HPm, in catalytic amounts, completely activates HPg to HPm, essentially all radioactivity is isolated in the SK*-HPm complex. If, as believed, SK* exchange to HPg is not sufficiently rapid, then the kinetic mechanism recently proposed by Kosow (21), and described above, cannot be a major mechanism for the activation of HPg by SK*-HPm. Whether very small amounts of HPm are generated at very early reaction times by this exchange mechanism cannot be conclusively evaluated; but, certainly no more than trace amounts of HPm can arise in this manner. We feel that SK* is simply too tightly bound to HPg or HPm to allow sufficient rapid exchange of SK*. Thus, we agree with earlier proposed mechanisms, which evoke SK*-HPm as an enzyme catalyzing HPg activation as the major means of converting HPg to HPm.

One final point can be made which is contradictory to the streptokinase exchange mechanism of Kosow (21). We find (Figs. 3 and 4) that SK* appears to be significantly more tightly bound to HPm than to HPg. Since this is so, it would seem that exchange of SK* from SK*-HPm to HPg would be unlikely. The kinetic SK* exchange mechanism requires this type of exchange to take place.
In conclusion, we present definitive evidence that the SK* exchange from SK*·HPm to HPg or to HPm does not occur at a sufficiently rapid rate to propose this step as a mechanistic event in HPg activation. We believe that SK*·HPm is an enzyme which serves as an activator of HPg.

REFERENCES

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