FURTHER EVIDENCE FOR AN ACTIVE CENTER IN STREPTOKINASE-PLASMINOGEN COMPLEX; INTERACTION WITH PANCREATIC TRYPSIN INHIBITOR*

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SUMMARY

Earlier work has shown that streptokinase and human plasminogen form a stoichiometric complex in which the presence of a functional active center can be detected by reaction with the active center-specific reagent, p-nitrophenyl-p'-guanidinobenzoate. The complex possesses activator activity, i. e. it catalyzes the conversion of plasminogen to plasmin. Evidence is presented to show that pancreatic trypsin inhibitor abolishes both the activator activity and the ability to react with the active center-specific reagent. This is accomplished, not by displacement of streptokinase, but by the formation of a ternary complex with streptokinase-plasminogen.

Human plasminogen can be activated to plasmin by the addition of the bacterial protein, streptokinase (SK)[1]. It is well established that SK forms a stoichiometric complex with plasmin and this complex has activator activity. It was also observed, however, that even when all plasmin activity in the plasminogen preparation had been blocked, SK was still able to activate plasminogen (1, 2). Recently it was shown that when SK forms a complex with plasminogen, an active center develops in the zymogen moiety, which is able to react with the active center-specific reagent, p-nitrophenyl-p'-guanidinobenzoate (NphBzoGdn) (3, 4). We have also shown that this com-

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[1] The abbreviations used are: SK, streptokinase; NphBzoGdn, p-nitrophenyl-p'-guanidinobenzoate; Tos-ArgOMe, tosylarginine methylester.

plex has activator activity towards human plasminogen, thus identifying it as the major activator in this system (4). The enzymatic properties of this complex differ from those of SK-plasmin, in that it is unable to hydrolyze casein, or Tos-ArgOMe. That the complex still contains plasminogen and not plasmin is shown by the finding that the peptide chain is still intact, while in plasmin an arg-val bond is cleaved during activation (5).

In the present paper we show that pancreatic trypsin inhibitor, which inhibits plasmin stoichiometrically, also inhibits SK-plasminogen. Inasmuch as the trypsin inhibitor reacts neither with SK, nor with plasminogen, by themselves, its reactivity toward the complex must be predicated upon a conformational change similar to that which also gives rise to the development of NphBzoGdn reactivity. That the trypsin inhibitor must be blocking the active center in the complex is shown by the observations that 1. it completely inhibits activator activity, and 2. it prevents reaction with the active center-specific reagent.

MATERIALS AND METHODS

Human plasminogen was purified as described earlier (4). Highly purified streptokinase ("Kabikinase", 10^5 Christensen U/mg) was the generous gift of Dr. Hugo Nihlen of AB Kabi, Sweden. Pure bovine pancreatic trypsin inhibitor (Kunitz type) was kindly donated by Dr. M. Laskowski, Sr. NphBzoGdn was from Nutritional Biochemicals; Tos-ArgOMe was from Mann Research.

Tos-ArgOMe esterase activity was determined spectrophotometrically according to Hummel (6). Titrations were done in 0.05 M Tris-HCl, pH 8.1, containing 1 mM substrate, by measuring the increase in absorbance at 247 nm in a Gilford 240 spectrophotometer.

Active site titration with NphBzoGdn was carried out at 25° according to

Chase and Shaw (7) in a Cary double beam recording spectrophotometer, using the 0-0.1 slide wire, at 410 nm. Both sample and reference cuvettes contained the desired components dissolved in 1 ml of 0.05 M phosphate, 0.01 M lysine, 0.1 M NaCl, pH 7.4.

Acrylamide gel electrophoresis in sodium dodecyl sulfate of reduced proteins was carried out according to Shapiro et al. (8). Gel chromatography was done using Sephadex G 200 (Pharmacia) equilibrated with 0.05 M Tris-HCl, 0.02 M lysine, 0.1 M NaCl, pH 8.0, in a 0.9 x 25 cm column, at room temperature.

RESULTS AND DISCUSSION

Effect of trypsin inhibitor on plasmin development. Since pancreatic trypsin inhibitor is known to react stoichiometrically with plasmin, it was of interest to see whether it will have any effect on the activation of plasminogen by SK. Plasmin development upon addition of equimolar SK was followed by Tos-ArgOMe hydrolysis, as shown in Fig. 1. When plasminogen and SK were mixed and 15 seconds later were added to substrate, plasmin activity developed gradually (curve b) and approached the activity exhibited by fully developed plasmin (curve a). In contrast, when SK was added to plasminogen in the presence of a two-fold excess of inhibitor and the mixture transferred 1 min later to substrate, no plasmin activity developed (curve c). Since the inhibition observed could have been due to either inhibition of activation or to inhibition of plasmin formed, a direct method for the determination of plasmin was employed.

Gel electrophoresis in sodium dodecyl sulfate clearly distinguishes between plasminogen and plasmin, as the former is a single peptide chain, while the latter consists of two chains after reduction (5). Fig. 2, lower row, shows the development of plasmin as a function of time in a solution of plasminogen,

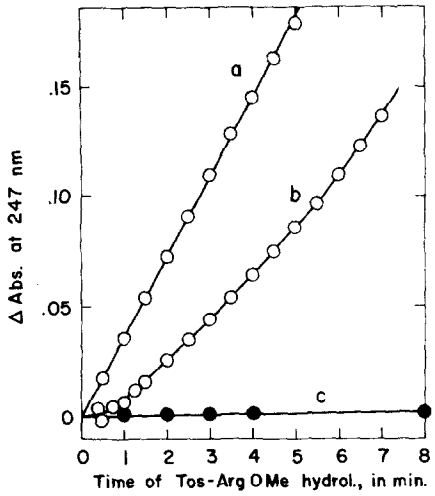


Fig. 1. Plasmin development in the presence and absence of trypsin inhibitor, tested by Tos-ArgOMe hydrolysis. Curve a: activity of fully developed plasmin. Plasminogen and SK mixed, and transferred to substrate 20 min later. Curve b: same, transferred to substrate 15 sec after mixing. Curve c: Plasminogen and SK mixed in the presence of twice-equivalent trypsin inhibitor, transferred to substrate 1 min after mixing. Components present in 3 ml of assay mixture: 85 μg plasminogen, 50 μg SK, and 12 μg trypsin inhibitor (only in c).

activated by an equimolar amount of SK, in the absence of trypsin inhibitor. It can be seen that the plasminogen band gradually disappears and two new bands, corresponding to the heavy and light chains of plasmin, accumulate. Also, the SK band undergoes the modification noted earlier (4). When, however, activation is carried out in the presence of a two-fold excess of inhibitor (upper row), no change in the pattern is observed: only plasminogen and unmodified SK are present. Thus, trypsin inhibitor effectively prevented SK activation of human plasminogen.

Nature of inhibited complex. To exclude the possibility that the inhibitor interfered with activation by displacing SK from plasminogen, gel filtration was carried out to determine the components in the reaction mixture. Fig. 3 shows that when SK was added to plasminogen in the presence of trypsin inhibitor, the protein peak (SK-Plgn-PTI) was eluted earlier than was

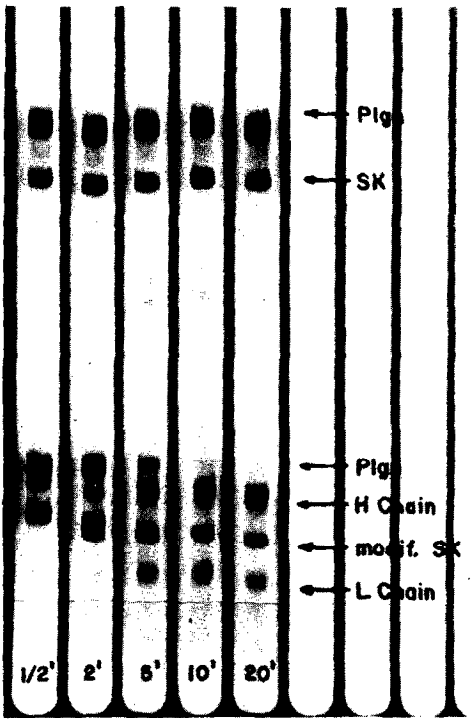


Fig. 2. Gel electrophoresis in sodium dodecyl sulfate of plasminogen activated by SK in the presence (upper row), and absence (lower row) of trypsin inhibitor. Aliquots of the activation mixtures were reduced and denatured at the times indicated on the Fig. Components applied to gel: 49 μg plasminogen, 25 μg SK, 5.6 μg trypsin inhibitor (only in upper row). Migration: from top to bottom. Plgn: plasminogen; SK: streptokinase; H chain: heavy chain; L chain: light chain; Modif. SK: modified streptokinase.

plasminogen alone (Plgn), indicating a larger molecular weight. The same elution profile was obtained when the inhibited complex of SK-plasmin was chromatographed (SK-Plmn-PTI). That this increase in apparent size must be due to the presence of SK in the complex, is shown by gel electrophoresis (Fig. 4) of the peak tubes from the two lower curves in Fig. 3. Both patterns show the presence of SK but the material which was activated in the presence of the inhibitor contains only plasminogen, while the control shows the two plasmin bands. (The faint band corresponding to the inhibitor cannot be seen on the photograph.) The presence of the inhibitor is, of course, also indicated by the fact that no activation has taken place. Thus, addition of SK

TABLE I

Effect of trypsin inhibitor on the reaction of p-nitrophenyl-p'-guanidinobenzoate with SK-plasminogen complex[a].

	Components[b]	p-nitrophenol liberated nmoles[c]	nmoles p-nitrophenol / nmoles Plgn
Expt. 1	Sample: Plgn, SK + NphBzoGdn Reference: Plgn + NphBzoGdn	2.50	0.72
Expt. 2	Sample: Plgn, PTI, SK + NphBzoGdn Reference: Plgn, PTI + NphBzoGdn	0.12	0.03
Expt. 3	Sample: Plgn, PTI, NphBzoGdn + SK Reference: Plgn, PTI + NphBzoGdn	1.61	0.46

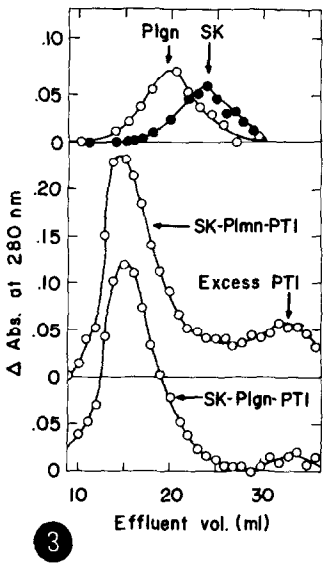
[a]For details of procedure, see Materials and Methods.

[b]Components in the sample and reference cuvettes which are listed preceding the + sign were first mixed; the component following the sign was added simultaneously to the two cuvettes, and recording was started within 5 seconds. In Expt. 2, SK was allowed to induce the active site for 15 seconds before NphBzoGdn was added. Components contained in the 1 ml reaction mixture were: 3.5 nmoles plasminogen (Plgn), 3.8 nmoles SK, 4.6 nmoles trypsin inhibitor (PTI), and 10 nmoles NphBzoGdn.

[c]Calculated from ϵ_{410}=16,595 (7).

to plasminogen in the presence of inhibitor results in the formation of a ternary complex between the three proteins.

Interaction with active site. That the inhibition of activation by trypsin inhibitor is due to interaction of the latter with the active site of the SK-plasminogen complex was demonstrated by using the active center-specific reagent, NphBzoGdn (Table I). When this reagent reacts with freshly formed SK-plasminogen, a burst of p-nitrophenol liberation is observed (Expt. 1). When SK is added to plasminogen in the presence of inhibitor, no burst is observed upon addition of the reagent (Expt. 2), indicating that the inhibitor



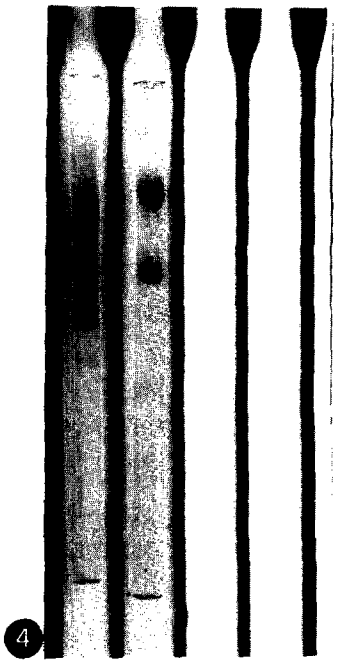


Fig. 3. Sephadex G 200 chromatography of the ternary complex. Top: 286 μg plasminogen (Plgn), and 500 μg streptokinase (SK). Middle: 570 μg plasminogen activated for 20 min by 360 μg of SK, then mixed with 90 μg of trypsin inhibitor (SK-Plmn-PTI). Bottom: 570 μg of plasminogen activated with 360 μg of SK in the presence of 90 μg of trypsin inhibitor. Fractions of 1 ml were collected at a flow rate of 20 ml/hour.

Fig. 4. Gel electrophoresis in sodium dodecyl sulfate of SK-plasmin-trypsin inhibitor complex (left), and of SK-plasminogen-trypsin inhibitor complex (right). Material for electrophoresis was obtained from the peak tubes in Fig. 3, after freeze drying. Left pattern (from top to bottom): heavy chain, native SK, modified SK (dark band), light chain. Right Pattern: plasminogen, native SK. Compare with gels in Fig. 2.

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must have blocked access of the reagent to the active center. In Expt. 3 the order of addition was reversed: SK was added as the last component. A sizable burst was observed, indicating induction of the active center by SK, despite the presence of trypsin inhibitor. Thus the reaction of the inhibitor with the active center was slower than that of the reagent. It is obvious therefore that when trypsin inhibitor reacts with SK-plasminogen the active center of the latter must already be present.

The fact that trypsin inhibitor interacts with the activator complex demonstrates that the conformation around the active center of the plasminogen moiety in the complex must already be similar to that in the fully developed plasmin, even though no chain cleavage has yet occurred. It seems therefore that a conformational change, similar to that suffered by plasminogen in the course of activation via cleavage of an arg-val bond, is accomplished here merely by distortion of the zymogen structure by the tightly bound SK molecule.

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