Regulation of Streptokinase–Human Plasmin Complex by the Plasma Proteinase Inhibitors α2-Antiplasmin and α2-Macroglobulin Is Species Specific and Temperature Dependent

By Steven L. Gonias, Nancy L. Figler, and Lela L. Braud

Streptokinase-plasmin complex (SkPI) was prepared with human plasminogen. Regulation of SkPI and plasmin by the plasminogen activator complex, α2-antiplasmin (α2AP) and α2-macroglobulin (α2M), was studied as a function of temperature in plasminogen-depleted human plasma, mouse plasma, and solutions of purified proteins. The reaction of plasmin with proteinase inhibitors in human plasma was complete. α2AP was the predominant inhibitor. The fraction of α2M-plasmin recovered was not affected significantly by incubation temperature. In contrast, the reaction of SkPI with human proteinase inhibitors was markedly temperature dependent. The apparent second-order rate constant for the reaction of SkPI with purified α2AP at 37°C (1.5 x 10^8 mol/L^-1 s^-1) was >150-fold higher than the constant derived at 4°C. In human plasma and in solutions containing mixtures of purified human proteins, α2AP was the principal inhibitor of SkPI. Elevating the temperature enhanced the reaction of SkPI with α2AP and α2M comparably. Equivalent results were obtained when incubations were performed in platelet-rich plasma (PRP) or whole blood. In murine plasma, SkPI reacted readily with the proteinase inhibitors. The principal inhibitor of SkPI was α2M. Maximum reaction between SkPI and murine α2M was observed at 37°C; however, significant reaction also occurred at 4°C. α2AP was the predominant inhibitor of plasmin in mouse plasma. Reaction of α2AP with SkPI in murine plasma was significant only after the α2M was inactivated with methylamine. These results were not affected by platelets or whole blood cells. We conclude that the thrombolytic efficacy of streptokinase reflects not only the nature of the plasminogen activator complex but also the function of the proteinase inhibitors.

© 1988 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Experimental Procedures

Reagents. H-D-Val-l-Leu-l-Lys-p-nitroanilide-HCl (S-2251) was purchased from Helena Laboratories (Beaumont, TX). p-Nitrophenyl-p'-guanidinobenzoate (PNPGB) and N-benzoyl-DL-arginine-p-nitroanilide-HCl were from Sigma (St Louis). Na^+Cl^- was from BDH (Toronto, Ont). Ribozyme buffer was from Burke Biologics (Burlington, Ont). Solutions were made up in 0.01 M sodium phosphate buffer, pH 7.4, unless noted.

Streptokinase-plasmin complex (SkPI) was prepared in 1 ml of 0.01 M sodium phosphate buffer, pH 7.4, by adding 1 μl of human plasminogen to 1 μl of human SkPI. The reagents were mixed, and the mixture was incubated for 10 min at 37°C. Plasminogen was depleted by the following method. A solution containing 5% bovine serum albumin, 0.1 M sodium phosphate buffer, pH 7.4, was incubated with 20 volumes of diazapeptin from Sigma (St Louis) at 37°C for 15 min. This solution was mixed with 1 volume of a solution containing 100 μg of human plasminogen, 100 μg of human fibrinogen, 25 μg of human fibrin monomer, and 25 μg of human fibrinogen fragment D. The mixture was incubated at 37°C for 60 min. The amount of plasminogen was measured by measuring the absorbance at 405 nm, and the activity of SkPI was determined by measuring the absorbance at 405 nm following the hydrolysis of p-nitroanilide-HCl.
from Amersham (Arlington, IL), and iodobeads were from Pierce (Rockford, IL). All other reagents were of the best grade commercially available.

**Proteins.** Streptokinase was purified from Kabikinase (Kabi, Stockholm) by the method of Castellino et al.\textsuperscript{14} The final preparation demonstrated a single band with an apparent mol wt of 50,000 by sodium dodecyl sulfate SDS-gel electrophoresis. Plasminogen was prepared from human plasma as described by Deutsch and Mertz\textsuperscript{15} and activated with catalytic quantities of streptokinase with 4% glyceral added or with <10 CTA U urokinase. \(\alpha_2\)AP was purified from human plasma by the method of Wiman.\textsuperscript{16} Human \(\alpha_2\)M was purified as previously described\textsuperscript{23} and was free of "reacted form" as judged by native gel electrophoresis.\textsuperscript{22} Anti-urokinase antibody was a gift of Dr Salvatore Pizzo (Duke University Medical Center). Fibrinogen, L grade, was purchased from Kabi and further purified by chromatography on lysine-Sepharose. Soybean trypsin inhibitor and trypsin were from Sigma. The trypsin was 60% active as determined by active site titration.\textsuperscript{23}

**Activity assays.** Plasmin activity after activation of purified plasminogen was determined by hydrolysis of S-2251 (0.8 mmol/L) under previously specified conditions.\textsuperscript{24} A Hewlett Packard 8450A temperature-controlled diode-array spectrophotometer was used (Hewlett Packard, Sunnydale, CA). Percentage of activity was calculated based on published steady-state kinetic parameters using a molar extinction coefficient of 9,950 for nitroaniline at 405 nm. Plasminogen in plasma preparations was assayed by a similar method after acid precipitation to remove inhibitors.\textsuperscript{24} \(\alpha_2\)M in solutions containing purified proteins and in plasma was assayed at 22°C with S-2251 by the method of Ganrot.\textsuperscript{27} \(\alpha_2\)AP activity was determined based on the depletion of S-2251 amidase activity in plasma or solutions containing plasmin. In these studies, plasminogen was activated with urokinase. The urokinase was then inactivated with \(\alpha_2\)M. Reaction with proteinase inhibitors in PPP was determined by this method. All experiments were performed in duplicate, and the results were averaged.

**Protease inhibitor distribution experiments.** SkPl or plasmin was prepared with radiolabeled plasminogen and incubated with various combinations of purified inhibitors, plasma, or modified plasma. Components were diluted in 20 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4, and incubated for 20 minutes unless otherwise specified. The temperature was varied. Reactions were terminated with 0.1 mmol/L PNPGB and analyzed using the electrophoresis-autoradiography procedure described above. All experiments were performed at least in duplicate.

**RESULTS**

**Effect of Temperature on the Regulation of Plasmin in Human Plasma**

Plasmin was incubated with variably diluted PPP at 4, 22, and 37°C. Solutions were then equilibrated at 22°C, and residual plasmin activity was determined using 0.8 mmol/L S-2251. In this system, substrate hydrolysis is catalyzed by the free protease and by plasmin bound to \(\alpha_2\)M at nearly equal rates; the \(k_{cat}\) and \(K_m\) values are 11.8 s\textsuperscript{-1} and 0.18 mmol/L for plasmin and 8.5 s\textsuperscript{-1} and 0.21 mmol/L for \(\alpha_2\)M-plasmin under these exact conditions.\textsuperscript{28} Figure 1 shows that when the quantity of \(\alpha_2\)AP in the PPP was sufficient to inhibit the added plasmin, amidase activity was decreased by 90% to 95%. The residual activity most likely represented plasmin bound to \(\alpha_2\)M. Temperature did not affect the distribution of the plasmin between \(\alpha_2\)AP and \(\alpha_2\)M as determined by this method.

Equivalent experiments were performed with radioiodinated plasmin. Reaction with protease inhibitors in PPP was studied at different temperatures using the somewhat more sensitive electrophoresis procedure (Fig 2). The relative mobilities of plasminogen, \(\alpha_2\)AP-plasmin, and \(\alpha_2\)M-plasmin are shown in the insets (Fig 2). Approximately 80% to 90% of the plasmin that reacts with \(\alpha_2\)M forms a covalent bond.\textsuperscript{28} This material distributes into two bands representing plasmin bound to two \(\alpha_2\)M subunits or four \(\alpha_2\)M subunits. A small correction factor was applied uniformly to the data to account for the fraction of noncovalent \(\alpha_2\)M-plasmin complex. At each of the temperatures studied, all of the active form characteristic of plasmin.\textsuperscript{28} This result indicates that the SkPl studied in this investigation was uniformly streptokinase-plasmin.

SkPl (0.2 \(\mu\)mol/L) was reacted with a sixfold molar excess of \(\alpha_2\)AP in 20 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4, at variable temperatures. At various times, aliquots of the reaction mixture were removed and the SkPl was inactivated with 0.1 mmol/L PNPGB. Samples were denatured without reductant and subjected to SDS-polyacrylamide gel electrophoresis on 5% slabs using the imidazole-HEPES (pH 7.3) buffer system described by McLellan\textsuperscript{26} as modified by Gonia et al.\textsuperscript{29} All gels were autoradiographed, and each lane of the dried gel was sliced into 3-mm sections and counted in an LKB model 1275 Minigamma \(\gamma\) counter (counting efficiency for \(^{125}\)I >75%). This procedure quantitates the plasmin in each gel band. Apparent second-order rate constants \((k_{app})\) were calculated using the basic equation:

\[
k_{app} = \frac{1}{[I]^{1/2} - [E]^{1/2}} \ln \left( \frac{[E]^{1/2} - [I]^{1/2}}{[I]^{1/2} - [E]^{1/2}} \right)\]

with \([I]^{1/2}\) and \([E]^{1/2}\) the initial concentrations of inhibitor and enzyme, respectively, and \([E]\) the concentration of enzyme at time \(t\). All experiments were performed in duplicate, and the results were averaged.
variably diluted PPP in mm (active enzyme concentration, 75 nmol/L) was incubated with 2251.

was calculated assuming that the plasma concentration of a2AP is 25°C (O), or 37°C (D). Estimated molar ratio of a2AP to plasmin l5.

compete for proteinase was observed at 4°C; however, the plasmin bound to a2M was much less than 10% at all temperatures.

Regulation of SkPl by Human Plasma Proteinase Inhibitors

The reaction of SkPl with purified a2AP was temperature dependent as expected. Data are summarized in Table 1. The kapp determined at 25°C was midway between the two previously reported values of 0.8 mol/L s⁻¹ and 1.4 x 10² mol/L s⁻¹. The reaction rate at 37°C was >150-fold higher than the rate at 4°C.

Fig 1. Plasmin activity after addition to human plasma. Plasmin (active enzyme concentration, 75 nmol/L) was incubated with variably diluted PPP in 20 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4, for ten minutes. Incubation temperature was 4°C (Δ), 25°C (O), or 37°C (□). Estimated molar ratio of a2AP to plasmin was calculated assuming that the plasma concentration of a2AP is 1.0 μmol/L. Residual plasmin activity was determined with S-2251.

plasmin reacted with the proteinase inhibitors. The role of a2AP was uniformly predominant, as was detected by sub

strate assay. A slight decrease in the ability of a2M to a2AP was uniformly predominant, as was detected by sub-

I .0 smoI/L. Residual plasmin activity was determined with 5-

pH 7.4, for ten minutes. Incubation temperature was 4°C (O), 25°C (O). Estimated molar ratio of a2AP to plasmin I:::

Table 1. Apparent Second-Order Rate Constants for Reaction of SkPl with a2AP

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>kapp (mol/L s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>1.5 x 10⁷</td>
</tr>
<tr>
<td>25</td>
<td>2.3 x 10⁶</td>
</tr>
<tr>
<td>15</td>
<td>3.4</td>
</tr>
<tr>
<td>4</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

The reaction of radioiodinated SkPl with proteinase inhibitors was studied in partially diluted PDP (Fig 3). The extent of reaction was markedly temperature dependent. At all temperatures, the major inhibitor of SkPl was a2AP. The distribution of radiolabeled proteinase between a2AP and a2M after incubation at 37°C approximated that observed in the plasmin experiments.

Platelets or whole blood cells were reconstituted in PDP as described. The radioiodinated SkPl distribution experiments were then repeated. The rate of reaction of the SkPl with the proteinase inhibitors and the fractions associated with a2AP and a2M remained unchanged.

Experiments performed with purified proteins confirmed that SkPl reacts preferentially with a2AP at 37°C (Fig 4). a2M-Proteinase complex was recovered at significant levels only when a2AP was not included in the incubation. At 4°C, most of the SkPl did not react with either proteinase inhibitor. Table 2 summarizes the data. Equivalent results were obtained when SkPl was formed with unpurified Kabikinase mixture.

Temperature-dependent dissociation of SkPl to regenerate active plasmin was considered as a possible explanation for the reaction of the enzyme with a2AP. Many previous studies indicate that dissociation does not occur. Studies of the murine system presented below strongly support non-dissociation. As additional evidence, fibrinogenolysis experiments

Fig 2. Covalent binding of plasmin to proteinase inhibitors in human plasma. ¹²⁵I-Plasminogen was activated with 5 U urokinase for ten minutes at room temperature and then treated with antitryptase antibody. Activity was 47% to 58% complete. The plasmin (0.17 μmol/L) was incubated with variably diluted PPP for ten minutes at 4°C (O, □), 25°C (O, □), and 37°C (Δ, △). Samples were analyzed by electrophoresis, autoradiography, and gel slicing. Closed symbols: percentages of a2AP-¹²⁵I-plasmin. Open symbols: percentages of a2M-¹²⁵I-plasmin. Insets a and b show autoradiography of unreacted plasmin (PI) and plasmin incubated at 37°C with PPP at an estimated molar ratio of a2AP to plasmin of 0.8, respectively.

Fig 3. Reaction of SkPl with proteinase inhibitors in human plasma. SkPl (0.17 μmol/L) was formed with ¹²⁵I-plasminogen and incubated with PDP diluted into 20 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4, for 30 minutes at 4, 25, and 37°C. Estimated molar ratios of a2AP to SkPl were calculated for each incubation mixture assuming that the concentration of a2AP in plasma is 1.0 μmol/L. Reactions were terminated with PNPGB and analyzed by electrophoresis. Percentage of recovery of total radioactive plasmin is shown for a2AP-plasmin (O) and a2M-plasmin (△) after incubation at 37°C; a2AP-plasmin (O) and a2M-plasmin (△) after incubation at 25°C; and a2AP-plasmin (□) after incubation at 4°C.
were performed (Fig 5). In these experiments, SkPI was incubated with plasminogen-depleted fibrinogen at 4 or 37°C. Terminal plasmic fibrinogen digestion products were not generated by the SkPI. The faint bands with slightly increased mobility as compared with fibrinogen indicate that the SkPI did cause limited proteolysis; however, this change was comparable at 4 and 37°C. That this mild proteolysis represented dissociated plasm is therefore highly unlikely.

**Regulation of SkPI by Murine Plasma Proteinase Inhibitors**

When human SkPI was incubated with murine plasma, significant reaction with α2M was observed (Fig 6). Radioactive α2M-plasmin was recovered in bands with apparent mol wts of 420,000 and >700,000, which corresponded closely with the human α2M-plasmin bands. The reaction was temperature dependent; however, even at 4°C, nearly 40% of the SkPI formed complex with α2M (Table 3). At 37°C, <20% of the radioactivity was recovered in the free plasin band. Because these data are not corrected for the fraction of α2M-plasmin that lacks a covalent bond, the reaction at 37°C may have been essentially complete.

α2AP competed poorly with α2M when SkPI was added to the murine plasma. This result was independent of temperature. Extraction of the plasminogen from murine plasma by chromatography on lysine-Sepharose did not affect either the extent of reaction of SkPI with the inhibitors or the distribution between α2AP and α2M. Equivalent results were also obtained when SkPI was incubated with murine PRP or whole blood.

**Table 2. Reaction of SkPI With Purified Human α2AP and α2M**

<table>
<thead>
<tr>
<th>Concentration (μmol/L)</th>
<th>Recov 125I-plasmin (%)</th>
<th>Free α2AP-PI</th>
<th>α2M-PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SkPI       α2M</td>
<td>α2AP</td>
<td>Temperature (°C)</td>
<td></td>
</tr>
<tr>
<td>0.2 0 0</td>
<td>37</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.2 0.6 0</td>
<td>37</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>0.2 0.6 0.6</td>
<td>37</td>
<td>49</td>
<td>44</td>
</tr>
<tr>
<td>0.2 0.6 1.2</td>
<td>37</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>0.2 0.6 1.8</td>
<td>37</td>
<td>47</td>
<td>50</td>
</tr>
<tr>
<td>0.2 0 0</td>
<td>4</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.2 0.6 0</td>
<td>4</td>
<td>99</td>
<td>0</td>
</tr>
<tr>
<td>0.2 0.6 0.6</td>
<td>4</td>
<td>96</td>
<td>3</td>
</tr>
<tr>
<td>0.2 0.6 1.2</td>
<td>4</td>
<td>96</td>
<td>3</td>
</tr>
<tr>
<td>0.2 0.6 1.8</td>
<td>4</td>
<td>96</td>
<td>3</td>
</tr>
</tbody>
</table>

Incubation time 30 minutes.
Experiments were performed to evaluate the role of \( \alpha_2 \)AP as a fibrinolysis inhibitor in murine plasma (Fig 7). Human \( ^{125}\text{I}-\text{plasminogen} \) was activated with catalytic amounts of urokinase or streptokinase and incubated with PPP. Reaction occurred nearly exclusively with \( \alpha_2 \)AP (>95%) confirming previous in vivo studies. Murine plasma was then diluted 1:2 with a solution of 400 mmol/L methylamine, 20 mmol/L Tris-HCl, titrated to pH 8.0, and incubated at room temperature for 40 minutes to inactivate the \( \alpha_2 \)M. A second aliquot of plasma was treated similarly except that methylamine was omitted. SkP1 reacted readily with \( \alpha_2 \)M and minimally with \( \alpha_2 \)AP in the control plasma. In methylamine-treated plasma, significantly enhanced reaction of SkP1 with \( \alpha_2 \)AP was detected. These data suggest that the predominant role of murine \( \alpha_2 \)M in regulation of human SkP1 reflects enhanced reactivity with \( \alpha_2 \)M and not decreased reactivity with \( \alpha_2 \)AP.

**DISCUSSION**

\( \alpha_2 \)AP is a member of the serpin proteinase inhibitor family that includes \( \alpha_2 \)-proteinase inhibitor and antithrombin-III. The reaction of \( \alpha_2 \)AP with plasmin involves at least two steps: a very rapid noncovalent association step followed by covalent bond formation. The active-site serine in plasmin is unable to deacylate after reaction with the \( \alpha_2 \)AP active-site Arg-Met peptide bond.

The reaction of \( \alpha_2 \)M with proteinase involves a mechanism very different from other inhibitors. Each of the four identical \( \alpha_2 \)M subunits has a centrally located sequence of peptide bonds that is highly susceptible to proteolysis. Reaction of a proteinase with one of these peptide bonds proceeds unhindered through the step of deacylation; however, the cleavage causes a major conformational change in the \( \alpha_2 \)M that traps the proteinase. Covalent bonds form variably between proteinase lysine groups and specialized thioester bonds in each \( \alpha_2 \)M subunit. The \( \alpha_2 \)M–proteinase complex is irreversible, even when no covalent bonds form.

Based on the difference in reaction mechanism, different factors evidently affect the reactivity of a proteinase with \( \alpha_2 \)AP and \( \alpha_2 \)M. One important consideration in \( \alpha_2 \)M reactions is proteinase size. Reaction of SkP1 with human \( \alpha_2 \)M occurs through facilitated transfer of the plasmin from streptokinase to the inhibitor. Covalent bonds form variably between proteinase lysine groups and specialized thioester bonds in each \( \alpha_2 \)M subunit. The high-energy intermediate involving a temporarily strained SkP1–\( \alpha_2 \)M tertiary complex was proposed to explain the temperature dependence of the SkP1–\( \alpha_2 \)M reaction.

The importance of temperature helped reconcile reported in vitro studies of the SkP1–human \( \alpha_2 \)M reaction; however, the extremely fast reaction of human SkP1 with murine \( \alpha_2 \)M in vivo remained incompletely explained. The data we present demonstrate markedly enhanced reactivity of SkP1 with murine \( \alpha_2 \)M as compared with human \( \alpha_2 \)M. This enhanced reactivity was evident in plasma and did not require cell-surface cofactor activity. The SkP1–murine \( \alpha_2 \)M reaction was temperature dependent; however, significant levels of reaction were detected even at 4°C.

With an electrophoresis procedure, second-order rate constants were derived for the reaction of purified human \( \alpha_2 \)AP with SkP1. The value derived at 25°C (23 mol/L⁻¹ s⁻¹) was midway between two previously reported values determined at the same temperature with a substrate assay. The kinetics experiments were performed to demonstrate the marked effect of temperature on the SkP1–\( \alpha_2 \)AP reaction. The constants were calculated based on covalent product which actually forms in the second step of the reaction following noncovalent association. The previously used substrate assays did not require covalent binding to detect complex. Because the rate constants we report are not significantly lower than the previous values, formation of an SDS-stable covalent bond is not the rate-limiting step in the \( \alpha_2 \)AP–SkP1 reaction.
The ratio of recovered $\alpha_2$AP-$^{125}$I-plasmin to $\alpha_2$M-$^{125}$I-plasmin remained nearly constant when SKPI was incubated in human plasma at different temperatures. This result suggests that increased temperature enhances the rate of reaction of SKPI with both inhibitors comparably.

The reaction of SKPI with $\alpha_2$M in mouse plasma was essentially complete. The reaction of SKPI with human $\alpha_2$AP was not. This result was observed in experiments with purified inhibitors and in human plasma. The initial rates of reaction between human $\alpha_2$AP and SKPI followed the principles of second-order kinetics closely. After slightly more than 50% of the SKPI had reacted with $\alpha_2$AP, the rate of complex formation decreased. These data may reflect heterogeneity in the SKPI preparation. Such heterogeneity could result from the internal digestion of streptokinase in streptokinase-plasmin complex. The data we present suggest that murine $\alpha_2$M is unique in its ability to bridge any heterogeneity in the SKPI and react with the entire preparation.

Muninoglobulin is a proteinase inhibitor found in murine plasma but not in human plasma. Proteinases that bind to muninoglobulin retain esterase activity, a property attributed to the $\alpha$-macroglobulin class of inhibitors. The mol wt of muninoglobulin is 180,000. An SDS-stable covalent complex of muninoglobulin with plasmin would have a maximum mol wt of 260,000. The complexes formed during incubation of SKPI with murine plasma were significantly larger than the hypothetical muninoglobulin-plasmin complex. We therefore conclude that muninoglobulin does not contribute to the regulation of SKPI in the mouse.

Plasminogens from different species differ significantly in ability to bind streptokinase and form activator complex. The SKPI complexes that do form demonstrate widely variable activity. In contrast, studies of the proteinase inhibitors, $\alpha_2$M and $\alpha_2$AP, have generally demonstrated conservation of function across species lines. The reaction of $\alpha_2$M with SKPI is an exception to the rule, perhaps because SKPI is not a natural mammalian proteinase. We conclude that the thrombolytic activity of streptokinase in a given species can reflect variability in proteinase inhibitors as well as plasminogen.

REFERENCES

11. Wiman B: On the reaction of plasmin or plasmin-streptokinase complex with aprotinin or $\alpha_2$-antiplasmin. Thromb Res 17:143, 1980
Regulation of streptokinase-human plasmin complex by the plasma proteinase inhibitors alpha 2-antiplasmin and alpha 2-macroglobulin is species specific and temperature dependent

SL Gonias, NL Figler and LL Braud