Kinetics of the reaction of streptokinase–plasmin complex with purified human and mouse α_2 -macroglobulin

Implications for mechanism

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Streptokinase–human plasmin complex (Sk–hPm) reacted rapidly with purified mouse α_2 -macroglobulin ($m\alpha_2M$) *in vitro* at 37 °C. Approx. 98 % of the plasmin in Sk–hPm bound covalently to at least one $m\alpha_2M$ subunit. Most of the streptokinase dissociated (95 %). The rate of Sk–hPm inactivation clearly depended on the $m\alpha_2M$ concentration. With 1.2 μM - $m\alpha_2M$, 50 % of the Sk–hPm (0.02 μM) reacted in less than 50 s. A double-reciprocal plot comparing pseudo-first-order rate constants ($k_{app.}$) and $m\alpha_2M$ concentration yielded a second-order rate constant of $2.3 \times 10^4 M^{-1} \cdot s^{-1}$ ($r = 0.97$). This value is an approximation, since Sk–hPm preparations are heterogeneous. Sk–hPm reacted with human α_2M ($h\alpha_2M$), forming α_2M –plasmin complex (98 % covalent). More than 99 % of the streptokinase dissociated. The rate of reaction of Sk–hPm with $h\alpha_2M$ did not clearly depend on inhibitor concentration. The $k_{app.}$ values determined with 0.6–1.2 μM - $h\alpha_2M$ were decreased 10–20-fold compared with $m\alpha_2M$. In order to study the effect of Sk–hPm heterogeneity on the reaction with α_2M , the proteinase was incubated for various amounts of time at 37 °C before addition of inhibitor. The enzyme amidase activity was maximal within 5 min; however, reaction of Sk–hPm with $m\alpha_2M$ or $h\alpha_2M$ was most extensive after 20 min and 2 h respectively. After incubation for more than 1 h, Sk–hPm acquired fibrinolytic activity, suggesting plasmin dissociation. Therefore the enhanced reaction of $h\alpha_2M$ with ‘older’ Sk–hPm preparations may have resulted in part from dissociated plasmin or ‘plasmin-like’ species. By contrast, the reaction of Sk–hPm with $m\alpha_2M$ was most rapid when the proteinase preparation was free of plasmin, indicating direct reaction of Sk–hPm with $m\alpha_2M$ as the only major mechanism. Finally, streptokinase–cat plasminogen complex reacted more extensively with $m\alpha_2M$ than with $h\alpha_2M$, suggesting that $m\alpha_2M$ may be a superior inhibitor with this class of plasminogen activators in general.

INTRODUCTION

Streptokinase is a bacterial protein that binds to human plasminogen or plasmin with high affinity, forming a 1 : 1 stoichiometric complex [1]. The conformation of the plasmin active site is changed by streptokinase, as is the substrate-specificity of the proteinase [2]. Streptokinase–human plasminogen (Sk–hPg) and streptokinase–human plasmin (Sk–hPm) activate plasminogen by cleaving a specific Arg–Val peptide bond [2–4]. Plasmin does not cleave this bond and therefore is incapable of activating other plasminogen molecules.

Sk–hPg undergoes several intramolecular changes after formation *in vitro* or in human plasma [5]. The streptokinase is cleaved at a number of sites and the plasminogen is converted into plasmin [2,6–8]. This heterogeneity is important, since the various Sk–hPg and Sk–hPm species differ in activity when factors such as fibrinogen or Cl^- are present [9]. Plasminogens from many species do not form an activator complex with streptokinase (mouse, rat), and those that do form (cat, dog) tend to have a lesser stability than Sk–hPm [10–12].

Streptokinase is widely used as an intravenous drug for therapy of thrombotic disorders [13]. Since the active

form of streptokinase is a proteinase, activity in the circulation is subject to regulation by the plasma proteinase inhibitors. Free plasmin in the blood is rapidly inactivated by proteinase inhibitors [14]. α_2 -Antiplasmin (α_2AP) is the primary inhibitor of plasmin [15]; however, under some conditions plasmin may also react with α_2 -macroglobulin (α_2M) [16]. By contrast, Sk–hPm reacts slowly with α_2AP [1,17,18]. At 25 °C the second-order rate constant is decreased 10⁵-fold [1,17].

Early investigations suggested that α_2M does not react with Sk–hPm at all [19]; however, when preformed Sk–hPm was injected intravenously into mice, the proteinase reacted rapidly with α_2M and was cleared from the circulation within 10 min [20]. The discrepancy between this study *in vivo* and prior investigations *in vitro* was partially explained by the large temperature coefficient for the reaction Sk–hPm with human α_2M ($h\alpha_2M$) [21]. Significant inhibition of Sk–hPm by $h\alpha_2M$ was observed at 37 °C, but not at the lower temperatures that were studied previously. Still, the reaction of Sk–hPm with $h\alpha_2M$ *in vitro*, even at 37 °C, was not rapid enough to account for the inactivation of Sk–hPm *in vivo* in mice.

Most recently, this laboratory studied the reaction of Sk–hPm with proteinase inhibitors in mouse and human

Abbreviations used: Sk, streptokinase; Pm, plasmin; Pg, plasminogen; α_2M , α_2 -macroglobulin; α_2AP , α_2 -antiplasmin; h (prefix), human; m (prefix), mouse; c (prefix), cat.

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plasma [22]. The results demonstrated a heretofore unrecognized species-specificity in the regulation of the plasminogen activator complex. α_2 AP was the primary inhibitor of Sk-hPm in human plasma; however, in mouse plasma most of the Sk-hPm reacted with α_2 M. When mouse plasma was treated with methylamine to inactivate the α_2 M, significant reaction of Sk-hPm with α_2 AP was demonstrated. These experiments suggested a difference in the Sk-hPm-inhibitory activities of mouse α_2 M ($m\alpha_2$ M) and $h\alpha_2$ M; however, since the studies were performed with plasma, factors such as fibrinogen may have affected the results. In the present investigation the inhibition of Sk-hPm was studied *in vitro* with purified human and mouse α_2 M preparations. A significant difference in the kinetics of these reactions is demonstrated. The reaction of Sk-hPm with $m\alpha_2$ M *in vitro* is sufficiently rapid to account for the neutralization of Sk-hPm in the circulation of mice. Finally, the data presented here suggest that different reaction mechanisms may be important in the regulation of Sk-hPm by $h\alpha_2$ M and $m\alpha_2$ M.

MATERIALS AND METHODS

Materials

p-Nitrophenyl *p*'-guanidinobenzoate hydrochloride and *N* $^{\alpha}$ -benzoyl-DL-arginine *p*-nitroanilide hydrochloride were purchased from Sigma Chemical Co. D-Valyl-L-leucyl-L-lysine *p*-nitroanilide hydrochloride (S-2251) was from Helena Laboratories. Na¹²⁵I was from Amersham, and Iodobeads were from Pierce Chemical Co.

Proteins

Streptokinase was purified from Kabikinase (Kabi Co.) by the method of Castellino *et al.* [2]. The final preparation demonstrated a single band with an M_r of 50 000 by SDS/polyacrylamide-gel electrophoresis. Plasminogen was prepared from human plasma as described by Deutsch & Mertz [23]. The four separate human plasminogen preparations studied in this investigation contained mixtures of carbohydrate variants I and II. Carbohydrate variant II was uniformly present in slight excess as determined by acid/polyacrylamide-gel electrophoresis [24]. Cat plasminogen was purified by the method of Deutsch & Mertz [23] and was free of contaminants or active plasmin as determined by SDS/polyacrylamide-gel electrophoresis. Human fibrinogen (L grade) was purchased from Kabi Co. Trace quantities of plasminogen were removed from the fibrinogen preparation by chromatography on lysine-Sepharose as previously described [25].

Sk-hPm was prepared by incubating radioiodinated human plasminogen with a 2-fold molar excess of streptokinase in 7% (v/v) glycerol for 20 min at 37 °C unless otherwise specified. Under these conditions the plasminogen was completely converted into plasmin as determined by SDS/polyacrylamide-gel electrophoresis. The streptokinase was also degraded into smaller fragments, the most prominent having an M_r of 37 000, as previously described [6–8]. Fig. 1 shows the changes in streptokinase-plasmin(ogen) that occurred when the complex was incubated at 37 °C for different periods of time under the conditions used here. The conversion of plasminogen into plasmin was complete within the first

Fig. 1. SDS/polyacrylamide-gel electrophoresis of streptokinase-plasmin(ogen) after incubation for various times at 37 °C

The activator complex was prepared as described in the text. All samples were denatured in the presence of dithiothreitol (7 mg/ml). Abbreviations: Pm-H and Pm-L, plasmin heavy chain and plasmin light chain respectively.

few minutes. The composition of streptokinase peptide fragments continued to change during the first 45 min of incubation. Subsequently the composition of peptides was relatively stable as determined by electrophoresis.

Streptokinase-cat plasmin complex (Sk-cPm) was prepared by incubating cat plasminogen with a 20-fold molar excess of streptokinase for 10 min, as described by Summaria *et al.* [12]. Trypsin and soya-bean trypsin inhibitor were purchased from Sigma Chemical Co. The concentration of active proteinase in trypsin preparations was determined by the method of Chase & Shaw [26]. $h\alpha_2$ M was purified from plasma by the procedure of Imber & Pizzo [27]. $m\alpha_2$ M was prepared from citrated whole blood obtained by cardiac puncture of anaesthetized CD-1 female mice (Charles River Laboratories). The procedure was comparable with that used for preparing $h\alpha_2$ M except that NiCl₂ was substituted for ZnCl₂ in the metal ion-chelate chromatography step. Purified preparations of $h\alpha_2$ M and $m\alpha_2$ M were studied by using SDS/polyacrylamide-gel electrophoresis and soya-bean-trypsin-inhibitor-resistant amidase activity assays [28]. α_2 AP was purified from human plasma by the method of Wiman [29].

Radioiodination

Human plasminogen, cat plasminogen and streptokinase were radioiodinated with Iodobeads as described by the manufacturer (Pierce Chemical Co.). Desalting was performed on Sephadex G-25 (Pharmacia). Specific radioactivities for human and cat ¹²⁵I-plasminogen ranged from 0.5 to 4.0 μ Ci/ μ g. The specific radioactivities of ¹²⁵I-streptokinase preparations were 0.8–1.0 μ Ci/ μ g.

Determination of protein concentrations

Absorption coefficients for determining concentrations of plasminogen, streptokinase and α_2 M are listed elsewhere [20].

Reaction of Sk-hPm with α_2 M

Sk-hPm (0.02 μ M) was formed with 125 I-labelled human plasminogen and allowed to react with different concentrations of $m\alpha_2$ M or $h\alpha_2$ M (0.2–2.0 μ M) in 20 mM-sodium phosphate buffer, pH 7.4, containing 150 mM-NaCl at 37 °C. At various times portions of the reaction mixture were removed, and the Sk-hPm was inactivated with 0.1 mM-*p*-nitrophenyl *p*'-guanidinobenzoate. Samples were denatured under non-reducing conditions in 1.0% (w/v) SDS by heating at 100 °C for 3 min and subjected to SDS/polyacrylamide-gel electrophoresis in 5% slabs with the imidazole/Hepes buffer system, pH 7.3, described by McLellan [30] as modified by Gonias & Figler [31]. α_2 M-proteinase was clearly resolved from non- α_2 M-bound 125 I-plasmin, which dissociated from streptokinase in SDS. Gels were autoradiographed, and each lane was sliced into 6 mm sections for counting of radioactivity in an LKB model 1275 Minigamma γ -radiation counter (counting efficiency greater than 75%). This procedure quantifies the free 125 I-plasmin and the 125 I-plasmin covalently bound to α_2 M for each incubation time. Approximate pseudo-first-order rate constants (k_{app}) were calculated from inhibition curves by applying linear regression to data points generated during the first 75% of the reactions. Since the Sk-hPm preparation consisted of multiple species, each potentially reacting with α_2 M at a different rate, the k_{app} values are presented as approximations for comparative purposes. Each concentration of $h\alpha_2$ M or $m\alpha_2$ M was studied in at least two separate experiments, and the results were averaged.

Determination of covalent binding

Sk-hPm (0.08 μ M) was formed with 125 I-plasminogen and incubated with $h\alpha_2$ M or $m\alpha_2$ M (0.8 μ M) for 45 min at 37 °C. The reactions were stopped with *p*-nitrophenyl *p*'-guanidinobenzoate. α_2 M-plasmin and non- α_2 M-bound proteinase were resolved by chromatography on an Ultrogel AcA-22 column (1.2 cm $\times$ 25 cm). The α_2 M-proteinase was then subjected to SDS/polyacrylamide-gel electrophoresis. Covalently bound plasmin was recovered in the low-mobility α_2 M bands when the gel was sliced and counted for radioactivity as described above.

Streptokinase incorporation

In the reaction of Sk-hPm with $h\alpha_2$ M, most of the streptokinase dissociates from the α_2 M-plasmin complex [20]. The most likely explanation for streptokinase dissociation is that the bulky mass of the entire Sk-hPm complex cannot be accommodated within the dimensions of the $h\alpha_2$ M 'trap' [21]. In order to evaluate this mechanism with both $h\alpha_2$ M and $m\alpha_2$ M, Sk-hPm was prepared with radioiodinated streptokinase. The 125 I-Sk-hPm was then incubated with a 10-fold molar excess of either $h\alpha_2$ M or $m\alpha_2$ M for 45 min at 37 °C. The reaction products were chromatographed on Ultrogel AcA-22. Streptokinase binding to α_2 M was determined by measuring the radioactivity co-eluted in fractions with the proteinase inhibitor [32].

Steady-state amidase assays

Different preparations of streptokinase-plasmin(ogen) complex were formed by incubating human plasminogen with a 2-fold molar excess of streptokinase for various periods of time at 37 °C. Hydrolysis of S-2251 (80–

800 μ M) by each preparation of enzyme (0.04–0.11 μ M) was studied with the use of a Hewlett-Packard 8450A diode-array spectrophotometer. The buffer was 20 mM-sodium phosphate buffer, pH 7.4, containing 150 mM-NaCl, and the temperature for substrate hydrolysis was 20 °C. Kinetic parameters were determined from Lineweaver-Burk plots. The correlation coefficients ranged from 0.93 to 1.00. Each set of kinetic parameters was derived by averaging the results of four to eight separate experiments.

Heterogeneity of streptokinase-plasmin(ogen) complexes

Human plasminogen was incubated with a 2-fold molar excess of streptokinase at 37 °C for 4.5 h. Portions were removed at various times, beginning at 30 s, and allowed to react with $m\alpha_2$ M or $h\alpha_2$ M. The final concentrations of streptokinase-plasmin(ogen) and α_2 M were 0.02 μ M and 0.4 μ M respectively. The time of incubation with α_2 M was either 10 s or 3 min at 37 °C. Reactions were terminated with 0.1 mM-*p*-nitrophenyl *p*'-guanidinobenzoate. The samples were denatured under non-reducing conditions and subjected to electrophoresis and slicing. The extent of reaction was determined by the amount of α_2 M- 125 I-plasmin complex formed.

Fibrinogenolysis experiments

Human plasminogen and a 2-fold molar excess of streptokinase were incubated for various periods of time at 37 °C. Each streptokinase-plasmin(ogen) preparation (0.01 μ M) was then incubated with fibrinogen (1.2 μ M) for 30 min at 37 °C. Formation of fibrinogen-degradation fragments Y, D and E was monitored by SDS/polyacrylamide-gel electrophoresis [33] and gel scanning with a Kontes fibre-optic densitometer.

RESULTS

Characterization of $m\alpha_2$ M

Since the $m\alpha_2$ M purification procedure utilized here differs from previously reported procedures [34,35], the final preparation was characterized by SDS/polyacrylamide-gel electrophoresis (Fig. 2) and by the Ganrot assay [28] (Fig. 3). The $m\alpha_2$ M electrophoresis bands identified under reducing and non-reducing conditions correspond well to those previously described [34,36]. The major $m\alpha_2$ M subunit was composed of two polypeptide chains (M_r 160 000 and M_r 34 000) compared with the $h\alpha_2$ M subunit, which consisted of a single 180 000- M_r polypeptide. A faint band with an M_r of 185 000 was also identified in the $m\alpha_2$ M preparation, consistent with the work of Hudson *et al.* [36]. This band may represent a second subspecies of $m\alpha_2$ M subunits [36]. $m\alpha_2$ M and $h\alpha_2$ M were treated with trypsin before electrophoresis. The predominant product of both reactions was a 90 000- M_r polypeptide, as previously described [34].

The trypsin-binding capacity of $m\alpha_2$ M was determined on the basis of the ability of the inhibitor to bind active trypsin and protect it from inactivation by soya-bean trypsin inhibitor (Fig. 3). Each 1 mol of $m\alpha_2$ M bound approx. 1.1 mol of trypsin, as determined from the slope of the linear regression for molar ratios under 1:1 (proteinase/inhibitor). Additional increases in soya-bean-trypsin-inhibitor-resistant amidase activity were appreciated as the proteinase/inhibitor ratio was increased from 1:1 to 2:1; however, the increments were

Fig. 2. Characterization of α_2 M by SDS/polyacrylamide-gel electrophoresis

α_2 M and α_1 M ($0.7 \mu\text{M}$) were incubated with and without trypsin ($1.4 \mu\text{M}$) at room temperature for 5 min. The samples were denatured under non-reducing and reducing conditions. The incubation mixtures were: lanes a, α_2 M alone; lanes b, α_2 M with trypsin; lanes c, α_1 M alone; lanes d, α_1 M with trypsin.

Fig. 3. Trypsin-binding capacity of α_2 M

α_2 M (33 nM) was allowed to react with trypsin (0 – 132 nM) in 100 mM -Tris/HCl buffer, pH 8.2, containing 10 mM - CaCl_2 for 15 min at room temperature. Soya-bean trypsin inhibitor (SBTI) ($1.0 \mu\text{M}$) was then added to each sample and allowed to react for 15 min. Each sample was incubated with 1.5 mM - N^α -benzoyl-DL-arginine p -nitro-anilide for 30 min at 37°C . Reactions were terminated by acidification. Absorbances were determined at 410 nm . Experiments were conducted in duplicate.

small (less than 20% of maximal activity), consistent with the results of a previous study [36]. These data may reflect the formation of some 2:1 proteinase- α_2 M complexes.

Reaction of Sk-hPm with α_2 M and α_1 M

Sk-hPm was formed with ^{125}I -plasminogen and allowed to react with α_2 M. When the products were purified by chromatography and analysed by electro-

Fig. 4. Reaction of Sk-hPm with excess α_2 M

Sk-hPm ($0.02 \mu\text{M}$) was allowed to react with increasing concentrations of α_2 M (0.2 – $1.2 \mu\text{M}$) for up to 10 min. All reactions were conducted at 37°C and analysed by SDS/polyacrylamide-gel electrophoresis. The α_2 M/Sk-hPm molar ratios were 10:1 ($\circ$), 20:1 ($\square$), 30:1 ($\triangle$), 40:1 ($\bullet$), 50:1 ($\blacksquare$) and 60:1 ($\blacktriangle$).

phoresis, 98% of the ^{125}I -plasmin in the α_2 M-plasmin complex was covalently bound to at least one inhibitor subunit. The equivalent result was obtained when Sk-hPm was allowed to react with α_1 M; covalent binding of the radiolabelled plasmin exceeded 98%.

The kinetics of inhibition of Sk-hPm ($0.02 \mu\text{M}$) by α_2 M (0.2 – $1.2 \mu\text{M}$) were studied at 37°C , by using the electrophoresis procedure that detects covalent complex (Fig. 4). Each reaction proceeded rapidly and was more than 80% complete in less than 10 min. The rate of reaction depended on the concentration of α_2 M; however, all of the inhibition graphs were somewhat curvilinear, most probably reflecting heterogeneity in the Sk-hPm preparation. In the plasma the concentration of α_2 M is 2.0 – $3.0 \mu\text{M}$ [14]. The reaction of Sk-hPm with $1.2 \mu\text{M}$ - α_2 M was 50% complete in less than 50 s. This reaction rate adequately accounts for the previously reported rapid neutralization of Sk-hPm in the circulation of mice.

The reaction of Sk-hPm ($0.02 \mu\text{M}$) with α_1 M (0.2 – $2.0 \mu\text{M}$) was comparatively slow (Fig. 5). Table 1 compares the approximate pseudo-first-order rate constants (k_{app}) for the inhibition of Sk-hPm by α_1 M and α_2 M. The reaction of Sk-hPm with α_2 M was 10–20 times faster than the reaction with α_1 M (inhibitor concentration 0.6 – $1.2 \mu\text{M}$). When concentrations of α_1 M less than $1.2 \mu\text{M}$ were studied, the rate of reaction did not clearly depend on the concentration of inhibitor. This result suggests that inhibition of Sk-hPm by α_1 M may involve a complex mechanism or more than one pathway.

Effects of Sk-hPm heterogeneity on the reaction with α_2 M

Since Sk-hPg undergoes numerous internal structural transitions after formation, each Sk-hPm preparation contains a mixture of species. These species cannot be

Fig. 5. Reaction of Sk-hPm with excess $h\alpha_2M$

Sk-hPm ($0.02 \mu M$) was allowed to react with $h\alpha_2M$ (0.2 – $2.0 \mu M$) for up to 20 min. All reactions were conducted at $37^\circ C$ and analysed by SDS/polyacrylamide-gel electrophoresis. The α_2M /Sk-hPm molar ratios were 10:1 ($\circ$), 20:1 ($\square$), 30:1 ($\triangle$), 40:1 ($\diamond$), 50:1 (∇), 60:1 ($\times$), 80:1 ($\circ$) and 100:1 ($+$).

Table 1. Approximate pseudo-first-order rate constants for the reaction of Sk-hPm with α_2M

The concentration of Sk-hPm was $0.02 \mu M$. All reactions were conducted at $37^\circ C$. Each $k_{app.}$ value was calculated from the average slope of the reaction curve, including all measurements taken before reaction was 75% complete.

Concn. of inhibitor (μM)	$10^3 \times k_{app.}$ with $m\alpha_2M$ (s^{-1})	$10^3 \times k_{app.}$ with $h\alpha_2M$ (s^{-1})	$k_{app.}$ ratio ($m\alpha_2M/h\alpha_2M$)
0.2	4.0	0.76	5.2
0.4	5.9	0.80	7.4
0.6	8.6	0.84	10.2
0.8	10.2	0.86	11.9
1.0	13.0	1.02	12.7
1.2	15.3	0.90	17.0
1.6	—	1.38	—
2.0	—	1.78	—

resolved; however, the relative proportion of each species will vary with time. The effect of Sk-hPm heterogeneity on proteinase activity was studied by incubating streptokinase and plasminogen at $37^\circ C$ for different periods of time up to 4.5 h. The enzyme preparations thus formed were compared for amidase activity, fibrinogenolytic activity and reactivity with α_2M .

After incubation of streptokinase and human plasminogen for only 30 s, partial amidase activity (based on hydrolysis of S-2251) was achieved ($k_{cat.}/K_m$ $3.6 \pm 1.0 s^{-1}$; K_m $0.43 \pm 0.20 mM$; $k_{cat.}/K_m$ $10.5 \pm 2.5 mM^{-1} \cdot s^{-1}$). Full amidase activity was detected at 5 min ($k_{cat.}$ $15.3 \pm 2.0 s^{-1}$; K_m $0.38 \pm 0.09 mM$; $k_{cat.}/K_m$ $49.5 \pm 5.8 mM^{-1} \cdot s^{-1}$). Further incubation of the Sk-Pm, for 20 to 150 min, caused

Fig. 6. Reaction of streptokinase-human plasmin(ogen) with α_2M after preincubation for various times at $37^\circ C$

Sk-hPm ($0.02 \mu M$) was allowed to react with $0.4 \mu M$ $h\alpha_2M$ for 3 min ($\square$), with $0.4 \mu M$ $m\alpha_2M$ for 3 min ($\circ$) or with $0.4 \mu M$ $h\alpha_2M$ for 10 s ($\triangle$). The independent variable was the time that streptokinase was preincubated with plasminogen before addition of an inhibitor. Experiments were performed in triplicate.

little or no change in amidase activity ($k_{cat.}/K_m$ 36.2 – $46.7 mM^{-1} \cdot s^{-1}$) until 180 min, when the $k_{cat.}$ was mildly decreased ($6.4 \pm 0.4 s^{-1}$) and the $k_{cat.}/K_m$ was $31.3 \pm 5.5 mM^{-1} \cdot s^{-1}$.

The inhibition of streptokinase-human plasmin(ogen) by α_2M and $h\alpha_2M$ was studied by measuring the formation of α_2M - ^{125}I -plasmin complex after reaction for 3 min (Fig. 6). The 5 min streptokinase/plasminogen preincubation time (before addition of inhibitor), which was sufficient to generate maximum amidase activity, did not yield a preparation that reacted optimally with either $m\alpha_2M$ or $h\alpha_2M$. For $m\alpha_2M$, reaction was most extensive after preincubation of the enzyme for 20 min. Longer incubation times yielded Sk-hPm preparations that were slightly less reactive with $m\alpha_2M$. By contrast, the reaction of Sk-hPm with $h\alpha_2M$ was optimal only after the streptokinase and plasminogen were incubated for at least 2 h. These results indicate that different Sk-hPg and Sk-hPm species may react preferentially with $m\alpha_2M$, $h\alpha_2M$ and S-2251.

It was previously demonstrated that plasmin is undetectable in Sk-hPm preparations incubated at $37^\circ C$ for up to 50 min [22]. Therefore, after preincubation of the proteinase for only 20 min, the inhibition of Sk-hPm by $m\alpha_2M$ most probably resulted from a direct bimolecular reaction involving the intact activator complex. The older Sk-hPm preparations that reacted most rapidly with $h\alpha_2M$ are less well characterized. When the inhibition of Sk-hPm by $h\alpha_2M$ was studied with a 10 s reaction time (instead of 3 min), it was apparent that most of the inhibition of the older enzyme was the result of a rapidly reacting proteinase species (Fig. 6). Fibrinogenolysis experiments were performed to determine if this rapidly reacting species could be dissociated plasmin. Streptokinase and plasminogen were incubated at $37^\circ C$ for various amounts of time and then added to solutions of fibrinogen for an additional 30 min. Fibrinogen-digestion products (Y, D and E) were not detected after preincubation of Sk-hPm for up to 20 min, confirming our previous findings (results not shown) [22]. When

Fig. 7. Reaction of Sk-cPm with $h\alpha_2M$ and $m\alpha_2M$

Sk-cPm was formed with ^{125}I -labelled cat plasminogen (lane a). The activator complex ($0.02 \mu M$) was allowed to react with $1.4 \mu M$ - $h\alpha_2M$ for 3 min (lane b), with $1.4 \mu M$ - $m\alpha_2M$ for 3 min (lane c), with $1.4 \mu M$ - $h\alpha_2M$ and $0.5 \mu M$ - α_2AP for 3 min (lane d), with $1.4 \mu M$ - $m\alpha_2M$ and $0.5 \mu M$ - α_2AP for 3 min (lane e), with $1.4 \mu M$ - $h\alpha_2M$ and $0.5 \mu M$ - α_2AP for 10 min (lane f) or with $1.4 \mu M$ - $m\alpha_2M$ and $0.5 \mu M$ - α_2AP for 10 min (lane g).

older Sk-hPm preparations were studied, fibrinogenolysis was observed. Only trace quantities of digestion products (primarily fragment Y) were detected when incubation with fibrinogen was initiated at 40 min; however, after preincubation for 90 min, Sk-hPm converted 13.2% of the fibrinogen into fragment Y and 20.8% into fragments D and E. These percentages increased regularly as the Sk-hPm preincubation time was prolonged further, indicating that plasmin or a 'plasmin-like' Sk-hPm species was generated after extensive incubation at $37^\circ C$. In addition, the data suggest that dissociated plasmin may have been responsible for a significant fraction of the reaction with $h\alpha_2M$ after incubation of Sk-hPm for 2–4.5 h.

Binding of streptokinase to α_2M

Sk-hPm was formed with ^{125}I -streptokinase and allowed to react with $m\alpha_2M$ for 45 min. Streptokinase binding was determined by chromatography. Reaction of the proteinase with $m\alpha_2M$ was essentially complete as determined in identical experiments with Sk-hPm formed with ^{125}I -plasminogen; however, only 5% of the ^{125}I -streptokinase bound. The same experiment was repeated with $h\alpha_2M$; only 0.5% of the streptokinase bound to $h\alpha_2M$. These experiments demonstrate that most of the streptokinase dissociates when Sk-hPm reacts with $h\alpha_2M$ or $m\alpha_2M$.

Reaction of Sk-cPm with $h\alpha_2M$ and $m\alpha_2M$

Mouse plasminogen does not form a streptokinase activator complex [10]. Other plasminogens bind streptokinase; however, the complexes are less stable than Sk-hPm [11,12]. Cat plasminogen was chosen for this investigation, since Sk-cPm is the most stable non-human activator complex characterized thus far. Sk- ^{125}I -

Table 2. Reaction of Sk-cPm complex with α_2M

The concentration of Sk-cPm was $0.02 \mu M$. The concentration of each inhibitor, when present, was: $h\alpha_2M$, $1.4 \mu M$; $m\alpha_2M$, $1.4 \mu M$; α_2AP , $0.5 \mu M$. The values indicate the percentage of radiolabelled plasmin bound to each inhibitor after reaction for the specified time.

Inhibitor	Reaction time (min)	Plasmin bound (%)	
		α_2M	α_2AP
$h\alpha_2M$	3.0	24	—
$m\alpha_2M$	3.0	54	—
$h\alpha_2M$, α_2AP	3.0	3	59
$m\alpha_2M$, α_2AP	3.0	24	44
$h\alpha_2M$, α_2AP	10.0	4	66
$m\alpha_2M$, α_2AP	10.0	28	47

cPm ($0.02 \mu M$) reacted with both $h\alpha_2M$ and $m\alpha_2M$ ($1.4 \mu M$), forming covalent complexes that were detected by electrophoresis and autoradiography (Fig. 7). The extent of reaction in 3 min was much greater with $m\alpha_2M$. This result was not attributable to a difference in the rate of covalent-bond formation in the α_2M -plasmin complexes, since covalent binding was nearly uniform when the reactions were carried to apparent completion (results not shown).

Since reaction of Sk-cPm with α_2M might reflect dissociated plasmin, studies were performed with mixtures of α_2AP ($0.5 \mu M$) and α_2M ($1.4 \mu M$). In control experiments with this mixture of inhibitors, cat plasmin bound predominantly to α_2AP (85–90%). Equivalent results were obtained with $h\alpha_2M$ or $m\alpha_2M$. α_2AP was also the primary inhibitor of Sk-cPm in experiments with $h\alpha_2M$ or $m\alpha_2M$; however, $m\alpha_2M$ bound a significantly greater percentage of the proteinase than did $h\alpha_2M$ (Fig. 7). The quantity of ^{125}I -plasmin recovered in each band is summarized in Table 2. At this time we cannot determine whether α_2AP reacted directly with Sk-cPm or with dissociated cat plasmin. By contrast, the significant degree of reaction of Sk-cPm with $m\alpha_2M$ in the presence of α_2AP can only reflect a direct interaction with the activator complex. These data suggest that $m\alpha_2M$ may be more reactive than $h\alpha_2M$ with streptokinase-plasminogen activator complexes in general.

DISCUSSION

$h\alpha_2M$ and other α -macroglobulins react rapidly with many different proteinases from all four major classes [37]. Included are some of the enzymes that function in haemostasis [38]. Interestingly, the major plasminogen activators, tissue-plasminogen activator, urokinase and Sk-hPm, react poorly with $h\alpha_2M$ [22,39,40]. This laboratory has focused on the reaction of α_2M with Sk-hPm, since early studies suggested that α_2M might be the primary Sk-hPm inhibitor *in vivo* [20]. It is now clear that regulation of Sk-hPm by the proteinase inhibitors is species-specific [22]. The results presented here clearly implicate differences in the α_2 -macroglobulins as a cause of the species-specificity. Using purified inhibitor preparations, we estimated that Sk-hPm reacts 10–20-fold faster with $m\alpha_2M$ than with $h\alpha_2M$. An estimate of the Sk-hPm half-life in plasma may be calculated on the

basis of the k_{app} values in Table 1. Assuming that only α_2 M reacts with Sk-hPm in the blood and that the concentration of α_2 M is 1.2 μ M (a low estimate), the half-lives are 45 s and 12.8 min in mouse and human plasma respectively (for Sk-hPm incubated for 20 min at 37 °C). The calculated half-life in mouse plasma is clearly short enough to account for the rapid neutralization of Sk-hPm *in vivo* in the mouse. The present investigation does not rule out the possibility that fibrinogen or other plasma factors affect the reaction of Sk-hPm with α_2 M *in vivo*; however, this hypothesis need not be raised to explain the previous mouse clearance experiments [20].

In the reaction of Sk-hPm with α_2 M, most of the streptokinase dissociates and does not bind to the inhibitor [20,21]. In the present investigation, slightly increased amounts of streptokinase were recovered in complex with α_2 M; however, the major product of the reaction was still α_2 M-plasmin and not α_2 M-Sk-hPm. There are two possible explanations for this reaction mechanism. The first is that the Sk-hPm complex is in rapid equilibrium with small quantities of dissociated streptokinase and plasmin. The reaction observed with α_2 M then might reflect only the dissociated plasmin. Alternatively, the Sk-hPm may react directly with α_2 M, causing conformational change in the inhibitor and formation of an internal proteinase binding site that cannot accommodate the mass of the entire Sk-hPm complex [21]. The evidence presented here and elsewhere [21,22] supports the latter explanation with the exception of Sk-hPm preparations incubated at 37 °C for more than 1 h. When Sk-hPm was incubated for less than 50 min, no fibrinogenolytic activity was detected. In addition, Sk-hPm reacted almost entirely with α_2 M in the presence of α_2 AP in mouse plasma [22]. Since mouse α_2 AP is a more rapid inhibitor of plasmin than is α_2 M, the reaction of Sk-hPm with α_2 M in plasma must involve the intact plasminogen activator complex.

The reaction of α_2 M with proteinases involves a number of distinct steps that may be resolved by kinetic analyses [31,38,41,42]. Reversible association of a proteinase with α_2 M is followed by 'bait-region' cleavage, which triggers conformational change and exposure of thioester bonds. In a previous investigation it was suggested that the reaction of α_2 M with Sk-hPm was slow because of the proteinase size [21]. This speculation was based on the assumption that the 'bait-region' is positioned somewhat internally in the structure of α_2 M so that access is unlimited to small proteinases (M_r 20000–40000) but restricted for large proteinases (M_r > 100000). Recent electron-microscopy studies suggest that the structure of native α_2 M is rather open and that the 'bait region' may not become partially buried until after conformational change [43,44]. If this is correct, sequence-specificity and not proteinase size may be important in determining reactivity with α_2 M. The enhanced rate of reaction of α_2 M with Sk-hPm, compared with α_2 M, may then be explained by dissimilarities in the 'bait regions' of the two inhibitors.

When streptokinase and plasminogen were incubated together for various periods of time, transformations in the proteinase complex were detected by electrophoresis. These transformations yielded proteinase preparations consisting of different proportions of the various Sk-hPg and Sk-hPm species. For both α_2 M and α_2 M, maximal reactivity in the Sk-hPm preparation developed after full enzymic activity was achieved (based on hydrolysis of

S-2251). The resistance of early forms of streptokinase-plasmin(ogen) to inhibition by α_2 M may result in a decreased rate of Sk-hPm plasma clearance, especially if this result applies to other inhibitors as well.

Many proteinase inhibitor reactions may be described by the following mechanism proposed by Kitz & Wilson [45]:

The basic difference between this mechanism and a simple bimolecular reaction ($E + I \rightleftharpoons EI$) is the existence of an intermediate: a dissociable enzyme-inhibitor complex. Evidence for an intermediate of this nature has been obtained in some studies of α_2 M [31,41,42]; however, different proteinases can react by distinct mechanisms with the same inhibitor [46]. A second-order rate constant for the reaction of an inhibitor with a homogeneous enzyme preparation may be calculated from a plot of $1/k_{app}$ versus $1/[inhibitor]$ (double-reciprocal plot) independently of whether the mechanism includes one or two steps [45]. For the reaction of Sk-hPm with α_2 M, the values presented in Table 1 yield a linear double-reciprocal plot and a second-order rate constant of $2.3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (correlation coefficient 0.97). This value is at best an approximation describing the overall reaction, since the original k_{app} values were also approximations and because the Sk-hPm is clearly heterogeneous. A similar second-order rate constant was determined from a direct plot of k_{app} versus $[\alpha_2 \text{M}]$. For the reaction of Sk-hPm with α_2 M, the k_{app} values did not show a clear dependency on inhibitor concentration, and, as expected, the double-reciprocal plot was not linear. This behaviour might be explained if the rate-limiting step in the reaction of Sk-hPm with α_2 M is the generation of a specific form of the proteinase. This internal transformation would be a first-order process independent of α_2 M concentration. The fibrinogenolysis experiments suggest that one internal Sk-hPm transition resulting in reaction with α_2 M may be dissociation; however, this mechanism was most probably significant only with relatively old Sk-hPm preparations. Even after incubation of Sk-hPm for 4 h at 37 °C it is most likely that the reaction with α_2 M reflects many separate proteinase species including dissociated plasmin and intact forms of Sk-hPm.

The rapid inhibition of Sk-hPm by α_2 M suggests that the efficacy of thrombolytic agents in different species may be significantly altered by the plasma proteinase inhibitors. The possibility that potent anti-thrombolytic proteinase inhibitors such as α_2 M may specifically limit fibrinogenolysis is a topic for further investigation.

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