Characterization of the Reaction of Plasmin with α_2-Macroglobulin: Effect of Antifibrinolytic Agents†

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ABSTRACT: The reaction of several plasmin derivatives with α_2-macroglobulin (α_2M) has been investigated. Titration experiments measuring conformational changes in α_2M, changes in the number of sulfhydryl groups available for titration with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and changes in the ability of α_2M to protect bound plasmin from inhibition by soybean trypsin inhibitor all suggested that between 1.3 and 1.5 mol of plasmin was bound per mole of inhibitor. Under experimental conditions where [plasmin] > [α_2M], the conformational change occurring in the inhibitor and thiol group appearance displayed biphasic kinetics. Examination of the extent of subunit cleavage by plasmin revealed that the rapid phase was associated with cleavage of approximately two to three of the four α_2M subunits, while cleavage of the remaining subunits occurred during the slow phase of the reaction. Binary (1:1) α_2M–plasmin complexes were prepared by reacting a large excess of α_2M with plasmin and purifying the resultant complex by immunoaffinity chromatography using a monoclonal antibody specific for a neoantigen on α_2M that is generated when the inhibitor reacts with proteases or with methylamine. Characterization of the purified complex revealed that two of the four subunits were cleaved, and the conformational change, measured by alterations in the fluorescence of 6-(*p*-toluidino)-2-naphthalenesulfonate (TNS), was approximately 50% of that measured for a 2:1 complex. Thus it appears that proteolysis and conformational alterations associated with the binding of 1 mol of plasmin to α_2M are limited to one of two functional units in the molecule. The association rate of Lys_{77}- and Val_{442}-plasmin with α_2M was examined under conditions where the concentration of α_2M exceeded that of plasmin, assuring that 1:1 complexes constitute the major product of the reaction. The association rates were $(1.34 \pm 0.60) \times 10^5$ M^{-1} s^{-1} and $(1.75 \pm 0.64) \times 10^5$ M^{-1} s^{-1} for Lys_{77}-plasmin and Val_{442}-plasmin, respectively. One millimolar ε-aminocaproic acid had no effect on the association rate while the presence of 12 μM histidine-rich glycoprotein (HRG) slightly reduced the association rate to 0.31×10^5 M^{-1} s^{-1}. However, these molecules greatly reduce the association of plasmin with α_2-plasmin inhibitor [Lijnen, H. R., Hoylaerts, M., & Collen, D. (1980) *J. Biol. Chem. 255*, 10214–10222], lowering the rate to a value comparable to that measured for the reaction of plasmin with α_2M. Thus, in the presence of HRG, α_2M may play a significant role in the inhibition of plasmin activity.

Human plasminogen is a circulating plasma zymogen that upon activation to plasmin functions in the fibrinolytic pathway, effectively degrading both fibrinogen and fibrin. Regulation of plasmin activity is critical since this enzyme is capable of cleaving a large number of plasma proteins. A number of molecules exist in plasma that are capable of inhibiting plasmin activity. These include α_2-plasmin inhibitor (Collen, 1976; Morio & Aoki, 1976; Mollertz & Clemmensen, 1976), α_2-macroglobulin (α_2M)[1] (Ganrot, 1967; Harpel & Mosesson, 1973), C1 inactivator (Harpel & Cooper, 1975), antithrombin III (Highsmith & Rosenberg, 1974), and α_1-proteinase inhibitor (Rimon et al., 1966). α_2-Plasmin inhibitor reacts with plasmin at a rapid rate and is therefore an extremely effective inhibitor of plasmin. Inherited deficiencies of this protein are associated with a severe hemorragic tendency (Aoki et al., 1979). These findings indicate that α_2-plasmin inhibitor is the most important physiological inhibitor of plasmin activity, and it is generally thought that plasmin interacts with other inhibitors such as α_2M only under circumstances when α_2-plasmin inhibitor is totally consumed.

However, studies by Harpel (1981) have suggested that this conclusion requires further evaluation. By developing an enzyme-linked immunosorbent assay for the measurement of plasmin complexed with α_2M and α_2-plasmin inhibitor, his studies demonstrated that α_2M–plasmin complexes were detectable when plasmin was added to plasma at levels well below those required to saturate α_2-plasmin inhibitor. Further, this work demonstrated that major differences in the distribution of plasmin between these two inhibitors occurred depending on whether plasmin was added to plasma or whether plasminogen was activated endogenously with urokinase. To date, the underlying mechanisms that regulate the distribution of plasmin between these two inhibitors remain unknown. In the same study Harpel (1981) proposed that histidine-rich glycoprotein (HRG) may modulate the distribution of plasmin between these two inhibitors.

The interaction of plasmin with α_2M has been investigated in a number of laboratories (Cummings & Castellino, 1984; Christensen & Sottrup-Jensen, 1984; Gonias & Pizzo, 1983a; Straight & McKee, 1982), and discrepancies exist concerning the extent of plasmin binding and number of α_2M subunits cleaved when plasmin associates with the inhibitor. To date, the effects of antifibrinolytic agents on the reaction have not

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[1] Abbreviations: α_2M, α_2-macroglobulin; α_2-PI, α_2-plasmin inhibitor; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; ε-ACA, ε-aminocaproic acid; HRG, histidine-rich glycoprotein; PPACK, D-Phe-Pro-Arg-CH_2Cl; kDa, kilodalton(s); SBTI, soybean trypsin inhibitor; Tris, tris(hydroxymethyl)aminomethane; Bz, benzoyl; BSA, bovine serum albumin.

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been explored. In order to understand mechanisms important in regulating the inhibition of plasmin, a study was initiated to examine the interaction of plasmin with α_2M and the effect of antifibrinolytic agents on this interaction. The results demonstrate that antifibrinolytic agents, including HRG, have little effect on the association rate of plasmin with α_2M, suggesting that under certain conditions α_2M is an important in vivo plasmin inhibitor.

Materials and Methods

Proteins. α_2M was prepared according to the method of Imber and Pizzo (1981). The amount of active α_2M was determined to be greater than 90% by measuring the number of sulfhydryl groups released during reaction with an excess of trypsin as described previously (Steiner et al., 1985) and by analyzing the protein on SDS–PAGE and nondenaturing PAGE. Trypsin was obtained from Calbiochem and was prepared in 1 mM HCl. Active-site concentration, determined by titration with NPGB (Chase & Shaw, 1970), was 0.70 mol/mol of protein. Thrombin was prepared as described by Fenton et al. (1977) as modified by Steiner et al. (1985) and typically had activities ranging from 2500 to 3000 NIH clotting units/mg and contained 0.80 mol of active site/mol of enzyme based on active-site titration with NPGB (Chase & Shaw, 1970). Plasmin was prepared according to Morris et al. (1981), and the active-site concentration was determined to be 0.60 mol/mol by titration with NPGB (Chase & Shaw, 1970). Val_{442}-plasminogen was prepared and purified as previously described (Powell & Castellino, 1980). ^{125}I-plasmin was prepared according to the procedure of Fraker and Speck (1978). Histidine-rich glycoprotein (HRG) was isolated according to Kiode et al. (1982) and displayed the characteristic two bands at 74 (native form) and 67 kDa (degraded form) on reduced SDS–PAGE. It was estimated that between 60% and 70% of the total intensity represented the 74-kDa band.

Protein Concentrations. These were determined spectrophotometrically by using the following values for $E^{1\%}_{280\ nm}$ and molecular weight, respectively: α_2M, 8.93 and 718 000 (Jones et al., 1972; Hall & Roberts, 1978); trypsin, 15.4 and 23 300 (Robinson et al., 1971; Walsh & Neurath, 1964); thrombin, 18.3 and 38 500 (Fenton et al., 1977); Lys_{77}-plasmin, 17.0 and 84 000 (Barlow et al., 1969; Robbins et al., 1975); Val_{442}-plasmin, 16.0 and 38 000 (Sottrup-Jensen et al., 1977); HRG, 5.85 and 75 000 (Heimburger et al., 1972; Lijnen et al., 1983). In this paper, the concentrations stated for all proteases and α_2M are based on their active concentrations.

Free Sulfhydryl Determinations. Sulfhydryl appearance was measured with a Perkin-Elmer Lambda-5 spectrophotometer equipped with a programmable thermostated cell. The temperature was maintained at 30 °C, and the slits were set at 4 nm. An aliquot of protease was added to the cuvette containing 1–2 μM α_2M with 50 μM DTNB in 50 mM HEPES/150 mM NaCl, pH 8.0. The reaction was monitored at 412 nm, and a molar extinction coefficient of 13 600 M^{-1} cm^{-1} was used (Ellman, 1959). The concentration of DTNB in the cuvette was sufficient to ensure that the reaction of free sulfhydryl with DTNB was not rate limiting. Control experiments with α_2M and DTNB ensured that DTNB does not induce the appearance of sulfhydryl groups, and control experiments with protease and DTNB demonstrated that the proteases studied do not react with DTNB. Titration of 2 μM α_2M with 0–5 μM plasmin was done in plastic semimicrocuvettes. After a 30-min incubation, each cuvette was adjusted to 30 μM DTNB and the absorbance measured at 412 nm.

Cleavage of the 180-kDa Subunit. α_2M (2.71 μM) was incubated with a 2-fold molar excess of plasmin at 30 °C in 50 mM HEPES/150 mM NaCl, pH 8.0. At selected time intervals, aliquots were removed, and the reaction was terminated by addition of *p*-nitrophenyl *p*-guanidinobenzoate. The sample was then boiled in SDS sample buffer containing 5% β-mercaptoethanol for 5 min, and 0.2 μg was applied on 8–25% gradient slab gels and subjected to electrophoresis and staining with Coomassie blue on a Pharmacia PhastSystem electrophoresis apparatus.

Fluorescence Measurements. Intrinsic fluorescence was monitored in a SPEX-fluorolog-2 or a SLM-8000 fluorometer. The excitation wavelength was 280 nm, and the emission wavelength was 340 nm. The excitation and emission bandpasses were 0.1 and 1.0 nm, respectively, for the SPEX-fluorolog-2 and 2 and 16 nm, respectively, for the SLM-8000. The reaction was initiated by adding an aliquot of plasmin to α_2M in a buffer of 50 μM HEPES/150 mM NaCl, pH 8.0. The contribution of plasmin to the increase in intrinsic fluorescence was measured separately and the effect subtracted from the curve. Measurements of TNS fluorescence were performed in a buffer of 50 mM HEPES/150 mM NaCl, pH 8.0. Excitation wavelength was 315 nm, and emission was monitored at 412 nm. Stoichiometric titrations of α_2M (1 μM) with plasmin (0–5 μM), using TNS to monitor the reaction, were performed by using a Perkin-Elmer MPF-4 fluorometer after a 30-min incubation (excitation = 320 nm, emission = 420 nm, slits = 16 nm). Two control samples, in which a 4-fold molar excess of trypsin was added instead of plasmin, were also measured.

Kinetics Measurements. (*a*) *Second-Order Conditions.* Kinetic measurements were performed at concentrations of α_2M (0.5–1.0 μM) that exceeded those of plasmin (0.2–0.5 μM) by at least 2-fold. The reaction was carried out in a buffer of 50 mM HEPES/150 mM NaCl, pH 8.0 and 25 °C. Complexes formed under these conditions were isolated by immunoaffinity chromatography (see below), and the moles of protease incorporated per inhibitor molecule was determined. In all cases, this value was less than 1 mol of protease/mol of α_2M. Further, the degree of subunit cleavage, thiol group appearance, and extent of the conformational change were examined. These studies confirmed that primarily 1:1 complexes are formed, and the data were analyzed by assuming a bimolecular and irreversible reaction:

$$I + E \xrightarrow{k} EI \tag{1}$$

where

$$\text{velocity} = kIE \tag{2}$$

where k is the second-order rate constant, $I = [\alpha_2M]$, and $E = [\text{plasmin}]$. Under conditions where $I_0 > E_0$, where I_0 is the initial $[\alpha_2M]$ and E_0 is the initial [plasmin], integration of eq 2 results in the expression given by Jencks (1962):

$$\frac{1}{I_0 - E_0} \ln \frac{E_0(I_0 - x)}{I_0(E_0 - x)} = kt \tag{3}$$

where t is time, and x is the concentration of the α_2M–plasmin complex at time t. In these experiments it was assumed that $x = E_0 - E = E_0(F - F_0)/(F_{max} - F_0)$, where F is the fluorescence at time t, F_0 is the fluorescence at time zero, and F_{max} is the fluorescence at infinite time. The data were fitted by either nonlinear regression analysis or by plotting $[1/(I_0 - E_0)] \ln \{E_0(I_0 - x)/[I_0(E_0 - x)]\}$ vs t, which yields a straight line with a slope of k.

(*b*) *Pseudo-First-Order Conditions.* The reaction was also measured under pseudo-first-order conditions where the concentration of plasmin was 0.01 μM while the concentration

of α_2M was varied between 0.1 and 0.4 μM. The reaction was carried out at 25 °C in a buffer of 50 mM HEPES/150 mM NaCl, pH 8.0, and was initiated by the addition of α_2M. At selected time intervals the reaction was stopped by the addition of 50 μM SBTI. Plasmin activity not inhibited by SBTI was taken as a measure of the amount of complex that had formed. Control experiments were performed to demonstrate that plasmin in complex with α_2M was not inhibited by SBTI. In addition, experiments measuring the time course of plasmin inhibition with the high levels of SBTI chosen ensured that the reaction was rapidly quenched upon addition of SBTI.

Binding of ^{125}I-Plasmin to α_2M. α_2M (2.1 μM) was incubated with a 0–5.5-fold molar excess of ^{125}I-plasmin in 50 mM HEPES/150 mM NaCl, pH 8.0, at room temperature. Following incubation for 60 min, plasmin was inhibited by addition of aprotinin and PPACK. After 30 min, 50-μL aliquots were mixed with 50 μL of the appropriate buffer and analyzed by SDS–PAGE (Laemmli, 1970) or by Tris–borate PAGE (Nelles et al., 1980). Following electrophoresis, the gels were stained, destained, sliced, and counted.

Activity Assays. The assay used was similar to that reported earlier for measuring thrombin binding to α_2M (Straight & McKee, 1982). α_2M (1.56 μM) was incubated with 0–7.80 μM plasmin in 50 mM HEPES/150 mM NaCl, pH 8.0 at room temperature. After 40 min, 20 μM SBTI was added to each sample and then incubated for an additional 40 min. An aliquot (40 μL) was removed from each sample (in triplicate) and added to wells of microtiter plates containing 160 μL of 0.3 mM S-2251 in 50 mM Tris/150 mM NaCl, pH 7.4. After a 60-min incubation, the absorbance at 405 nm was determined with a Dynatech plate reader.

Immunoaffinity Purification of 1:1 α_2M–Plasmin Complexes. α_2M–plasmin (1:1) complexes were prepared by incubating 2 μM α_2M with 0.4 μM plasmin for 1 h at room temperature. The complex was purified by immunoaffinity chromatography using a monoclonal antibody specific for α_2M–protease complexes as described earlier (Steiner et al., 1986; Steiner et al., unpublished results). The purified monoclonal antibody was coupled to Affi-Gel 10 at a ratio of 2 mg/mL resin, and the column was found to have a capacity of 0.6 mg of α_2M–protease complex/mL of resin. The column was equilibrated with a buffer of 20 mM Tris/0.5 M NaCl, pH 7.4, prior to use. Native α_2M was not bound to the column, while α_2M–protease complexes and methylamine-treated α_2M were bound to the affinity column. These derivatives could be quantitatively recovered by elution with 20 mM sodium acetate, pH 5.0, containing 0.5 M NaCl. Immediately after elution, the pH was readjusted to 7.4. The ability of the purified binary α_2M–plasmin complex to bind trypsin was examined by incubating the complex with a molar excess of trypsin and separating bound trypsin from free trypsin by gel filtration chromatography. Trypsin activity was measured with the chromogenic substrate Bz-Ile-Glu-(γ-OR)-Gly-Arg-*p*-nitroanilide (S-2222, where R = CH_3 or H). The hydrolysis of this substrate by plasmin was minimal and was subtracted from the data used to calculate the amount of trypsin bound.

RESULTS

Stoichiometry of the Reaction of Plasmin with α_2M. A number of discrepancies exist in the literature concerning the reaction of plasmin with α_2M. These discrepancies include the extent of plasmin binding to the inhibitor and the number of α_2M subunits cleaved when plasmin associates with the inhibitor (Christensen & Sottrup-Jensen, 1984; Gonias & Pizzo, 1983a; Straight & McKee, 1982). In order to determine appropriate conditions for measuring the rate constants of the inhibition reaction, studies were initiated to investigate some of these discrepancies.

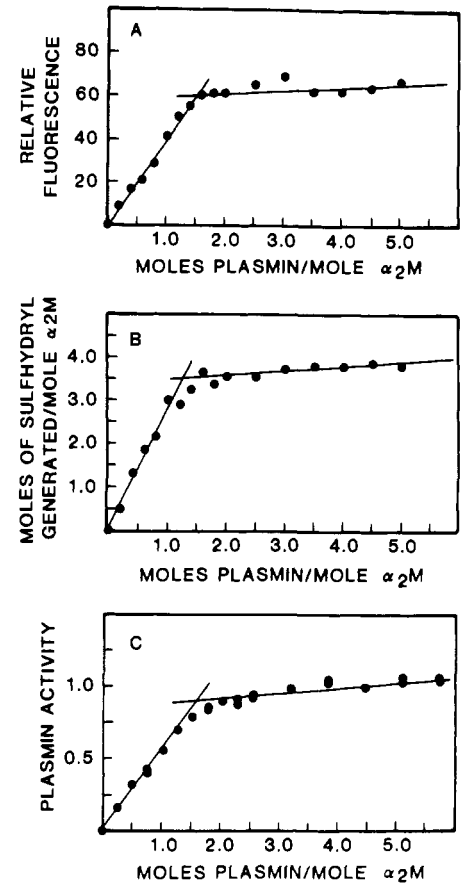

FIGURE 1: Titration of α_2M with increasing levels of plasmin. Increasing amounts of plasmin were reacted with fixed levels of α_2M for 40 min at room temperature in 50 mM sodium phosphate/150 mM NaCl, pH 7.4. (A) Changes in TNS fluorescence; [α_2M] = 1 μM; [TNS] = 50 μM. (B) Titration of available sulfhydryl groups with DTNB; [α_2M] = 2 μM; [DTNB] = 50 μM. (C) Measurement of bound plasmin activity. Following incubation, samples were mixed with a 10-fold molar excess of soybean trypsin inhibitor, and residual plasmin activity was measured by using the substrate D-Val-Leu-Lys-*p*-nitroanilide (S-2251); [α_2M] = 1.56 μM.

The amount of plasmin capable of binding to α_2M was measured by titration experiments. Three parameters, all measuring different properties of α_2M that are associated with proteolysis of the inhibitor, were monitored in these experiments. The properties that were measured include changes in the conformation of α_2M observed with the probe TNS (Figure 1A), changes in the number of sulfhydryl groups available for titration (Figure 1B), and changes in the ability of α_2M to protect bound plasmin from inhibition by soybean trypsin inhibitor (Figure 1C). In all titration experiments the concentration of α_2M remained fixed at levels between 1 and 2 μM. The stoichiometry determined by all techniques is between 1.3 and 1.5 mol of plasmin bound/mol of α_2M. These values are in agreement with those previously reported (Straight & McKee, 1982; Christensen & Sottrup-Jensen, 1984) and are slightly higher than those reported by Gonias and Pizzo (1983a).

The experiments described in Figure 1 all measured changes occurring in the inhibitor as a consequence of complex formation. In order to measure direct binding of plasmin to α_2M, various concentrations of ^{125}I-plasmin were incubated with fixed levels of α_2M for 1 h, and the complex was subjected to native PAGE to separate free from bound plasmin. The gels were sliced and counted, and the extent of binding was

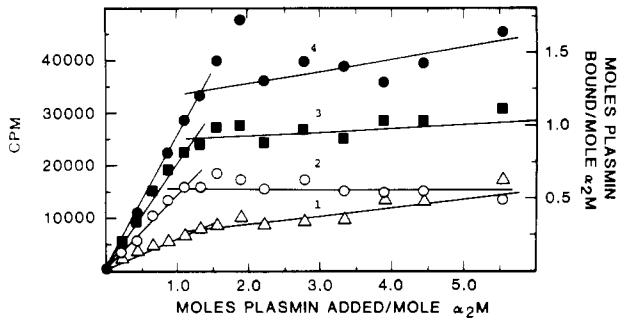

FIGURE 2: Determination of amount of ^{125}I-plasmin bound to α_2M. α_2M was incubated with increasing amounts of ^{125}I-plasmin for 60 min at room temperature in 50 mM HEPES/150 mM NaCl, pH 8.0. The complex was subjected to PAGE under nondenaturing (curve 4) and denaturing (curves 1–3) conditions. Curve 1: ^{125}I-plasmin located at the >180-kDa region following SDS–PAGE of the complex under reducing conditions. Curve 2: ^{125}I-plasmin located at the 140-kDa region following SDS–PAGE of the complex under reducing conditions. Curve 3: Total amount of ^{125}I-plasmin associated with α_2M polypeptides following SDS–PAGE of the complex under reducing conditions. Curve 4: ^{125}I-plasmin associated with α_2M following nondenaturing PAGE.

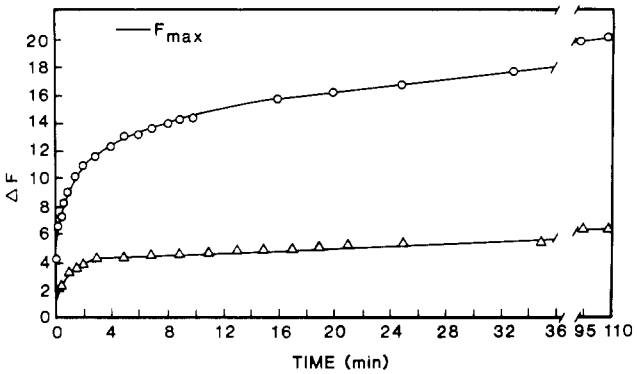

FIGURE 3: Time course of changes in TNS fluorescence occurring when plasmin reacts with α_2M. α_2M (final concentration = 1 μM) was incubated with plasmin at two different concentrations [final concentrations = 0.51 (Δ) and 2.02 (O) μM] at 30 °C in the presence of 50 μM TNS. The reaction was monitored continuously, and data points were taken at selected times from the trace. F_{max} shows the level of fluorescence that is obtained when α_2M is reacted with a 2-fold molar excess of trypsin. The buffer was 50 mM HEPES/0.15 M NaCl, pH 8.0. Excitation wavelength was 315 nm, and the emission was monitored at 412 nm. Ten-nanometer band-pass excitation and emission slits were utilized.

determined from the known specific activity of the original plasmin. The results of this experiment are shown in Figure 2 (curve 4). It is apparent that a break in the titration curve occurs at approximately 1.2–1.3 mol of plasmin/mol of α_2M, in excellent agreement with titration studies described earlier. A positive slope above this point was noted and suggests that additional plasmin can bind as the mole ratio of enzyme to inhibitor is increased and could represent nonspecific binding of plasmin with the inhibitor.

The extent of covalent binding was measured in a similar experiment in which the complex was subjected to SDS–PAGE under reducing conditions. Following slicing of the gel, counts were detected in four areas: ≥180, 140, and 60–80 kDa and at the dye front. The counts at the 60–80-kDa region and at the dye front were subsequently shown to represent the heavy and light chains of the free plasmin, respectively. Material in the 140-kDa region accounted for the majority of counts, and the amount of labeled plasmin located in this region is shown in Figure 2 (curve 2) while the amount of ^{125}I-plasmin located in the region that is ≥180 kDa is shown in Figure 2 (curve 1). The total extent of covalent binding was determined by summing counts in these two regions as shown in Figure 2 (curve 3). A comparison of curve 3 with curve 4 suggests that approximately 75% of the plasmin bound to α_2M is covalent, which is excellent agreement with the value reported earlier by Gonias and Pizzo (1983a). Other work has reported that approximately 54% of plasmin bound to α_2M is covalent (Salvesen & Barrett, 1980). However, the experiments were performed at a pH of 7.5, while those in the current study were performed at a pH of 8.0, which might be expected to alter the degree of covalent incorporation. Alternately, it is possible that loosely bound plasmin could dissociate upon nondenaturing electrophoresis of the complexes. The result of titration experiments in the present study confirms that approximately 1.5 mol of plasmin is bound per mole of α_2M and is consistent with the titration curves measuring changes in several properties of the inhibitor (Figure 1). These results corroborate those of Christensen and Sottrup-Jensen (1984), who postulated that some 2:1 complexes do form at higher mole ratios of plasmin:α_2M.

Conformational Changes Occurring in α_2M upon Reaction with Plasmin. Previous work has shown that the fluorescent dye TNS is a useful and sensitive probe for monitoring conformational changes occurring in α_2M (Strickland & Bhattacharya, 1984; Strickland et al., 1984; Steiner et al., 1985). The increase in fluorescence that occurs in this extrinsic probe when plasmin reacts with α_2M is shown in Figure 3. In the two separate experiments, the concentration of α_2M was maintained at 1 μM and the concentration of plasmin varied. When α_2M is incubated with an excess of plasmin, the reaction appears to be biphasic (Figure 3, circles), with the more rapid phase followed by a much slower process that eventually reaches the same fluorescence intensity as that seen when α_2M is reacted with a 2–3-fold molar excess of trypsin (F_{max}, Figure 3). Under the experimental conditions employed, no interaction of TNS with plasmin was detected. Further, TNS does not inhibit the amidolytic activity of plasmin, and the dye was found to interact rapidly with the preformed α_2M–plasmin complex to give a reproducible and stable fluorescence signal. Similar biphasic reactions were observed when the appearance of sulfhydryl groups was monitored by titration with DTNB (data not shown). In a second series of experiments, the concentration of α_2M exceeded that of plasmin. A biphasic reaction was also observed in this case (Figure 3, triangles), although the rate of the slow phase was reduced when compared with that of the previous experiment.

In order to investigate the correlation of TNS changes with plasmin binding, an experiment was performed in which aliquots were removed from the reaction mixture and incubated with a 20-fold molar excess of SBTI, and the residual plasmin activity was measured. This assay measures the amount of plasmin that becomes incorporated within the inhibitor (Ganrot, 1967). In this experiment, the concentration of α_2M was 1.0 μM, while the plasmin concentration was 0.51 μM. The results of this experiment, shown in Figure 4, indicate that the initial portion of the TNS fluorescence change parallels the association of plasmin with α_2M. However, the slow increase in TNS fluorescence does not appear to be associated with additional binding of plasmin since the amount of plasmin incorporated remains constant after 5-min incubation. Thus it appears that all of the plasmin is complexed with α_2M following the fast phase of the reaction. The mechanism giving rise to this slow increase in fluorescence is unknown. Since the concentration of α_2M exceeds that of plasmin in this experiment, presumably all of the plasmin is in complex with the inhibitor. Control experiments have shown that the fluorescence signal of TNS in the presence of native α_2M

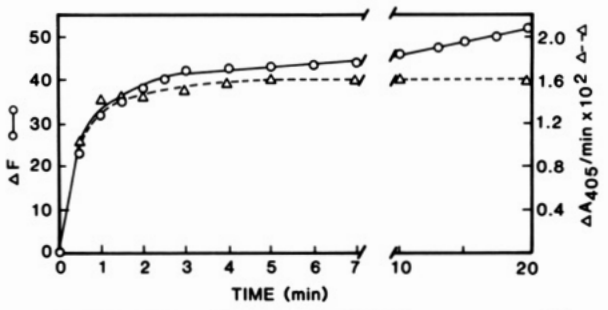

FIGURE 4: Correlation of changes in TNS fluorescence and incorporation of plasmin into the α_2M–plasmin complex. α_2M (1.0 μM) was incubated with plasmin (0.51 μM) in the presence of 50 μM TNS, and the changes in fluorescence were monitored at 412 nm (○). At selected time intervals, aliquots were removed from the cuvette and reacted with a 20-fold molar excess of SBTI for 2 min at room temperature. The sample was stored on ice and assayed for residual plasmin activity by using the substrate D-Val-Leu-Lys-*p*-nitroanilide (△).

remains constant over the time course of the experiment in Figure 3. Roche and Pizzo (1987) reported that bound plasmin could cleave α_2M subunits in an extremely slow reaction that was monitored over the period of days. Whether this slow increase in signal represents additional cleavage of subunits by bound plasmin remains to be determined.

Extent of Subunit Cleavage by Plasmin. Previous work examining the cleavage of α_2M subunits by plasmin has reported conflicting results. Straight and McKee (1982) found that approximately 75% of the subunits can be cleaved upon reaction with plasmin. Roche and Pizzo (1987) found that the extent of cleavage was dependent upon the amount of plasmin present during the reaction. Approximately half of the α_2M subunits were cleaved at a 2.5-fold molar excess of plasmin, while approximately 86% of the subunits were cleaved at a 20-fold molar excess of plasmin. Christensen and Sottrup-Jensen (1984) found that 27% of the subunits were cleaved at α_2M:plasmin mole ratios of 1:1 and 50% were cleaved at mole ratios of 1:3. In the present study, the extent of α_2M subunit cleavage was examined under two sets of conditions. In the first series of experiments, α_2M was reacted with excess plasmin (α_2M:plasmin 1:2) under conditions identical with those described for the experiments in Figure 3. At selected time intervals, the reaction was inhibited and the degree of subunit cleavage monitored by SDS–PAGE under reducing conditions. The results of these experiments (Figure 5A) verify that greater than 50% of the 180-kDa band, representing intact α_2M, disappears rapidly, with the concomitant appearance of major bands at 90–100 kDa. Additional proteolysis of the subunits occurs at a relatively slow rate, with approximately 80–90% cleaved at the final time point of 2 h. These results not only demonstrate that extensive cleavage of α_2M subunits by plasmin is possible but also demonstrate that the slower phase of the TNS fluorescence changes observed when [plasmin] $\geq 2[\alpha_2\text{M}]$ (see Figure 3) is correlated with additional proteolysis. Thus, despite the fact that only small amounts of ternary (1:2) complexes are formed during the reaction, plasmin appears capable of cleaving all four subunits of the inhibitor. This "nonproductive" cleavage in which subunit cleavage occurs in the absence of additional protease incorporation into the complex has been detected in a previous study examining the reaction of immobilized trypsin with α_2M (Björk, 1984).

The subunit cleavage pattern was also examined when 0.4 μM plasmin was reacted with 2 μM α_2M. At these ratios of inhibitor to enzyme, binary (1:1) complexes are the primary product of the reaction. Following incubation of plasmin with

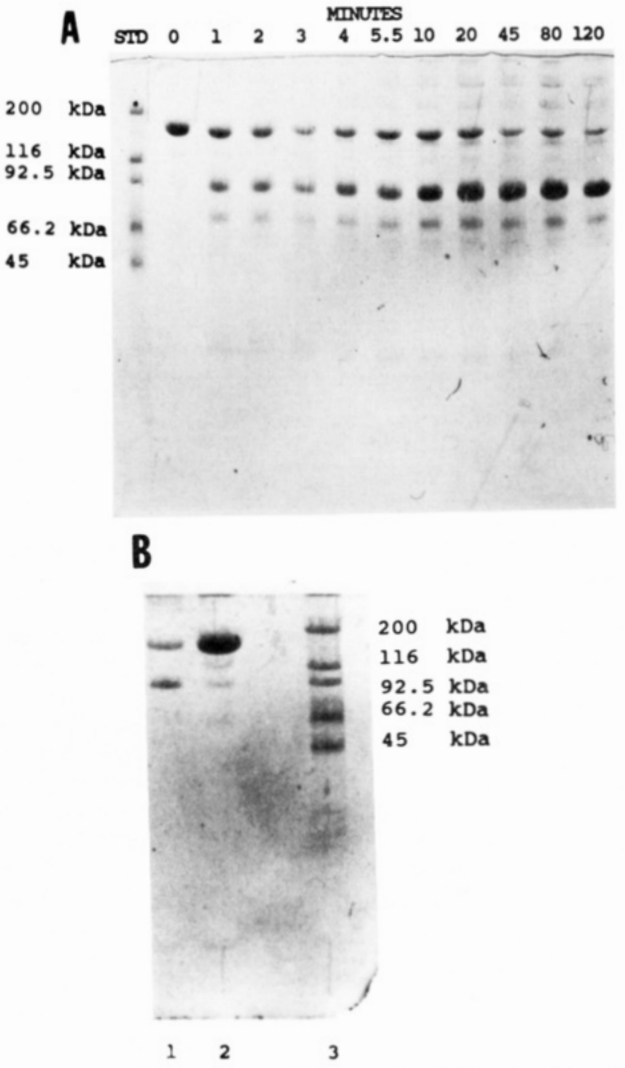

FIGURE 5: (A) Time course of the reaction of plasmin with α_2M analyzed by SDS–PAGE. α_2M (1 μM) was reacted with plasmin (2 μM) under conditions identical with those described in Figure 3. At indicated time intervals, an aliquot was removed and the reaction was inhibited with NPGB and boiled for 5 min in SDS sample buffer. Samples (0.2 μg) were applied to a Pharmacia PhastSystem electrophoresis apparatus. Standards (STD) include myosin, β-galactosidase, phosphorylase *b*, BSA, and ovalbumin. (B) SDS–PAGE under reducing conditions of purified binary α_2M–plasmin complex (lane 1) and native α_2M (lane 2). Samples (0.3 μg) were applied to a 8–25% gradient gel and run on a Pharmacia PhastSystem electrophoresis apparatus. Standards (lane 3) include myosin, β-galactosidase, phosphorylase *b*, BSA, and ovalbumin.

α_2M for 30 min at room temperature, the α_2M–plasmin complex was separated from native α_2M by immunoaffinity chromatography using a monoclonal antibody specific for a neoantigen on α_2M reacted either with proteases or with methylamine (Steiner et al., 1985; Steiner et al., unpublished results). Elution from this column is achieved by using a sodium acetate buffer, pH 5.0. Control experiments have substantiated that proteases do not dissociate from the inhibitor at this pH, and activity measurements confirmed that the purified complex contained 0.7 mol of plasmin/mol of α_2M. The isolated complex was analyzed by SDS–PAGE under reducing conditions, and the results are shown in Figure 5B. It is apparent that the association of a single plasmin molecule with α_2M results in the cleavage of half of the α_2M subunits, as evidenced by the disappearance of approximately 50% of the 180-kDa species and the appearance of 90–100-kDa species. Identical results were obtained when the α_2M–plasmin complex was reacted with diisopropyl fluorophosphate prior

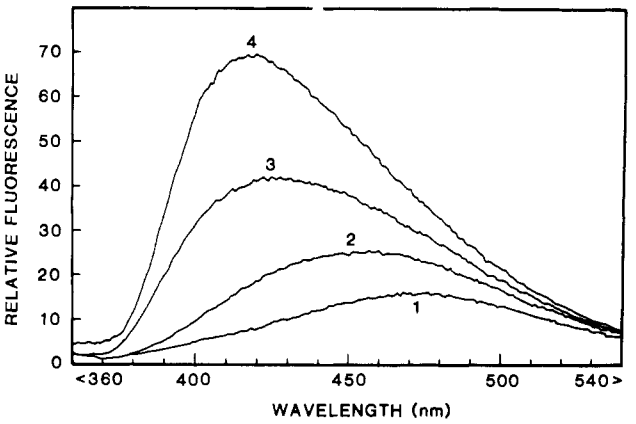

FIGURE 6: Emission spectra of TNS in the presence of (1) buffer, (2) 0.5 μM native α_2M, (3) 0.5 μM binary α_2M–plasmin complex, and (4) α_2M–trypsin complex (0.5 μM α_2M, 1 μM trypsin). The final concentration of TNS was 50 μM, and all scans were carried out at room temperature in 50 mM HEPES/150 mM NaCl, pH 7.4. Excitation was at 315 nm.

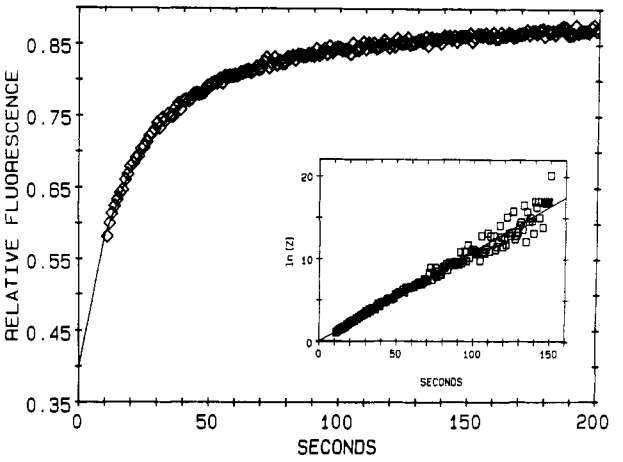

FIGURE 7: Representative data for the reaction of Lys_{77}-plasmin with α_2M monitored by changes in TNS fluorescence. Excitation was at 315 nm, and emission was monitored at 412 nm. Excitation and emission slits were 4 nm. Data were collected at 1-s intervals, and the reaction was carried out at 25 °C in a buffer of 50 mM HEPES/150 mM NaCl, pH 8.0. The concentration of α_2M was 0.52 μM, while the concentration of plasmin was 0.35 μM. The curve represents the best fit of the data to second-order kinetics determined by nonlinear regression analysis. Inset: Representation of the data in the linear form for a second-order plot, where $Z = [1/(I_0 - E_0)] \ln \{E_0(I_0 - x)/[I_0(E_0 - x)]\}$.

to isolation, and thus additional cleavage of α_2M subunits by bound plasmin was not detected during the relatively short period required to isolate the complex.

TNS Binding by the Isolated α_2M–Plasmin Binary Complex. Christensen and Sottrup-Jensen (1984) concluded that formation of 1:1 α_2M–plasmin complexes leads to a complete conformational change of the entire molecule. These studies employed changes in intrinsic fluorescence to monitor this process. On the other hand, Roche and Pizzo (1987) demonstrated that α_2M–plasmin complexes with two and four subunits cleaved differed considerably in their rate of in vivo clearance in a mouse model, implying that functional differences exist between these complexes. In the present studies, changes in TNS fluorescence were used as a probe to examine the extent of the conformational changes occurring in the purified binary (1:1) α_2M–plasmin complex. Figure 6 demonstrates the fluorescence of TNS in the presence of buffer (curve 1), native α_2M (curve 2), purified binary α_2M–plasmin complex (curve 3), and α_2M that had reacted with excess trypsin to form a ternary complex (curve 4). In the binary α_2M–plasmin complex, the level of fluorescence is not as extensive as that observed for the ternary α_2M–trypsin complex. Under the experimental conditions chosen, the fluorescence signal is directly dependent upon α_2M–protease concentration. A trace virtually identical with curve 4 was obtained when 2 mol of plasmin were incubated with 1 mol of α_2M for extensive time periods, or when α_2M was reacted with methylamine. Previous work examining the fluorescence of TNS in the presence of α_2M–protease complexes prepared by reacting α_2M with excesses of plasmin and trypsin (Strickland & Bhattacharya, 1984) has shown that the fluorescence is independent of proteases used to form the complex and is virtually identical with that of α_2M reacted with methylamine. The implications of the present data are that the reaction of plasmin with α_2M results in changes that affect one of two functional units in the whole molecule. A similar conclusion was obtained from data examining the reaction of thrombin with α_2M (Steiner et al., 1985).

The results of these experiments suggested that the purified binary α_2M–plasmin complex could bind additional proteases. This was further examined by incubating the complex with an equimolar amount of trypsin, isolating the complex by immunoaffinity chromatography, and measuring the amount of trypsin present as described under Materials and Methods. The results of this experiment demonstrated that the α_2M–plasmin complex was able to bind 0.4 mol of trypsin/mol of complex.

Kinetics of the Reaction of Plasmin with α_2M. The titration data presented earlier suggest that, at higher mole ratios of plasmin:α_2M, some 1:2 complexes do indeed occur. Thus the reaction of plasmin with α_2M under conditions of large protease excess is expected to be complicated, with the final reaction mixture containing both 1:1 and 1:2 α_2M–plasmin complexes. Kinetic measurements were therefore performed under conditions where the plasmin concentration was less than that of the inhibitor. These conditions ensure that binary complexes constitute the major product of the reaction and minimize the possibility of nonproductive proteolysis of α_2M by plasmin. The reaction was monitored by measuring changes in extrinsic (TNS) or intrinsic (tryptophan) fluorescence that are associated with conformational alterations occurring in the inhibitor upon proteolysis (Strickland & Bhattacharya, 1984; Björk & Fish, 1982; Straight & McKee, 1982) and by examining plasmin activity following incubation of the complex with SBTI.

Representative data of the kinetics of the reaction of Lys_{77}-plasmin with α_2M are shown in Figure 7. The changes in extrinsic fluorescence as well as intrinsic fluorescence (data not shown) reveal that the fluorescence approaches a maximum value. However, examination of the reaction for longer time periods demonstrates that an extremely slow increase in the TNS fluorescence does occur (see Figure 3). This slow increase does not affect the rate constants derived from the data for the first 200 s, and at the concentration of plasmin and α_2M used in these experiments, an excellent fit to second-order kinetics was noted (Figure 7). F_0 and F_{max} were both derived from the fitting procedure. The curve in Figure 7 displays the best fit to second-order kinetics, and the inset is a representation of the data in the linear form of a second-order plot. The rate constants determined for the reaction of this plasmin derivative and other plasmin derivatives with α_2M are summarized in Table I. When the concentrations of the initial reactants were varied, from 0.26 to 0.52 μM α_2M, while maintaining the same α_2M:plasmin ratio, no effect on the rate constant was noted. Further, the ratio of α_2M:plasmin

Table I: Effect of ϵ-ACA and HRG on Association Rate of Plasmin with α_2-Macroglobulin

enzyme	k_a (M^{-1} s^{-1})	n
Lys_{77}-plasmin	$(1.34 \pm 0.60) \times 10^5$	16
Val_{442}-plasmin	$(1.75 \pm 0.64) \times 10^5$	4
Lys_{77}-plasmin + 1 mM ϵ-ACA	$(1.26 \pm 1.05) \times 10^5$	10
Val_{442}-plasmin + 1 mM ϵ-ACA	$(1.75 \pm 0.69) \times 10^5$	4
Lys_{77}-plasmin + 12 μM HRG	$(0.31 \pm 0.17) \times 10^5$	3

was altered and identical results were obtained.

The effect of ϵ-aminocaproic acid (ϵ-ACA) on the reaction rate was measured for both Lys_{77}-plasmin and Val_{442}-plasmin. Concentrations of ϵ-ACA were high enough to completely saturate the high-affinity lysine binding sites of Lys_{77}-plasmin (K_d = 9 μM; Markus et al., 1978) yet low enough not to affect the catalytic activity of the enzyme. The results of these studies, summarized in Table I, demonstrate that this antifibrinolytic agent has no effect on the inhibition rate of either plasmin derivative with α_2M.

Since histidine-rich glycoprotein (HRG) is also capable of binding to plasmin(ogen) via the lysine binding sites, the effect of this protein on the inhibition rate was also measured. HRG was found to bind TNS, and thus this probe could not be used to monitor the progress of the reaction. Further, the high concentrations of HRG used in these experiments (10 μM) interfered with intrinsic fluorescent measurements. Consequently, the following assay was devised to monitor the progress of the reaction. Plasmin was preincubated with HRG for 2 min and the reaction initiated by adding α_2M. At selected time intervals, aliquots were withdrawn and the reaction was quenched by addition of a 476-fold molar excess of SBTI to inhibit any plasmin not in complex with α_2M. These high levels of SBTI were utilized to ensure a rapid quenching of the reaction. The residual plasmin activity, which is a measure of the amount of complex formed, was determined by measuring the hydrolysis of the chromogenic substrate S-2251. Control experiments were performed to ensure that SBTI at these levels rapidly inhibits all plasmin activity and does not inhibit plasmin when complexed with α_2M. This assay permitted evaluation of the kinetics under pseudo-first-order conditions. A plot of the pseudo-first-order rate constants measured at various concentrations of α_2M vs the concentration of α_2M was linear, and the association rate for Lys_{77}-plasmin with α_2M, derived from the slope of the line, was 1.10×10^5 M^{-1} s^{-1}. This value is virtually identical with that determined under second-order conditions with TNS as a probe to monitor the reaction (Table I). The rate constant of 0.31×10^5 M^{-1} s^{-1} obtained for the reaction in the presence of HRG is approximately 4-fold slower than the reaction in the absence of this antifibrinolytic protein. In order to determine if HRG altered the binding stoichiometry of plasmin with α_2M, titration experiments were performed in the presence and absence of this molecule. The results of these experiments revealed that HRG did not effect the binding stoichiometry of plasmin with α_2M.

Plasmin has been reported to degrade HRG (Smith et al., 1985; Lijnen et al., 1983) and consequently could cleave the molecule into fragments no longer capable of binding to the plasmin lysine binding sites. Thus an experiment was performed to measure the extent of cleavage occurring during the 5 min that is required to complete the kinetic measurements. The results of this experiment demonstrated (data not shown) that a small peptide is first cleaved from the 74-kDa species producing a 67-kDa species within 5–10 min of incubation. With time, more extensive cleavage of the molecule occurs. These results are similar to those reported by Smith et al. (1985). Lijnen et al. (1983) demonstrated that both the native (78 kDa) and degraded molecules (67 kDa) have a similar affinity for the lysine binding site(s) of plasmin(ogen). Thus cleavage of HRG that occurs during the kinetic measurements is largely restricted to conversion of some of the 78-kDa species to the 67-kDa species, which does not destroy the ability of HRG to bind to the plasmin(ogen) lysine binding sites.

Discussion

It is apparent that the reactions of trypsin, thrombin, and plasmin with α_2M share several common features. Under conditions of a 2–3-fold molar excess of protease, the reaction follows biphasic kinetics (Christensen & Sottrup-Jensen, 1984; Steiner et al., 1985). Similar time-dependent reactions have been observed both for the generation of thiol groups and for conformational changes that occur when a protease reacts with the inhibitor. In the case of either thrombin (Steiner et al., 1985) or plasmin (present study), examination of the extent of subunit cleavage with time reveals that the slower phase is associated with a reaction in which additional α_2M subunits are cleaved and some additional binding of plasmin occurs. Despite the fact that subunit cleavage goes to near-completion for either protease, less than 2 mol of protease is bound per mole of α_2M. Thus some nonproductive cleavage of α_2M must be occurring, in which additional α_2M subunits are cleaved, but the enzyme is not incorporated into the complex. It should be pointed out that cleavage by complexed enzyme could account for some of this nonproductive cleavage. However, it is likely that cleavage by the bound enzyme results in only a small fraction of the nonproductive cleavage, since Roche and Pizzo (1987) reported that the cleavage of α_2M subunits by bound plasmin occurs extremely slowly, over a period of days.

In addition to the difference in the extent of nonproductive cleavage by these different enzymes, the binding stoichiometry for each protease is different. It has been postulated that the binding stoichiometry of a protease for α_2M is related to the rate at which the protease reacts with a α_2M (Howell et al., 1983; Straight & McKee, 1984), and as the association rate increases, the binding stoichiometry approaches 2.0 mol of protease bound per mole of inhibitor. For example, 1 mol of α_2M will bind 1 mol of thrombin or 2 mol of trypsin when the inhibitor is incubated with a 2–3-fold molar excess of protease (Steiner et al., 1985; Straight & McKee, 1984; Howell et al., 1983; Sottrup-Jensen et al., 1980). The rate constant of 3.2×10^3 M^{-1} s^{-1} reported for the association of thrombin with α_2M (Steiner et al., 1985) is 6000-fold less than the rate constant of 2×10^7 M^{-1} s^{-1} reported for the association of trypsin with α_2M (Christensen & Sottrup-Jensen, 1984). The association rate of 1.3×10^5 M^{-1} s^{-1} measured for the reaction of plasmin with α_2M in the present study is intermediate between the rates for these two enzymes. Titration experiments indicate that between 1.3 and 1.5 mol of plasmin bind per mole of α_2M, indicating that the final product contains mixtures of both 1:1 and 1:2 complexes.

At this time, the relationship between the association rate of an enzyme with α_2M and its binding stoichiometry is not well understood. It has been proposed (Howell et al., 1984) that once an enzyme reacts with α_2M, the molecule undergoes a rapid conformational change that precludes further protease binding. According to this proposal, only those enzymes capable of reacting rapidly with α_2M prior to the conformational alterations occurring in the inhibitor could bind with a stoichiometry approaching 2. In order to evaluate this concept, more information is required concerning the nature of binary α_2M–protease complexes, a presumed intermediate in the

reaction sequence (Christensen & Sottrup-Jensen, 1984). The availability of a monoclonal antibody specific for α_2M–protease complexes has facilitated the isolation of binary α_2M–plasmin complexes. These complexes were prepared by reacting plasmin with a large excess of α_2M, which assured that binary complexes are the major product. The finding that this complex contains two intact subunits and conformational alterations that are approximately 50% of those measured for ternary complexes suggests that the conformational change is limited to one of two proposed functional units of the inhibitor. Studies are currently under way to further characterize binary complexes formed from different proteases in order to more fully understand the mechanism of the protease reaction with α_2M.

The extent of the conformational change measured in purified binary α_2M–plasmin complexes is interesting in view of recent reports (Ney et al., 1985; Roche & Pizzo, 1987) that the in vivo clearance of α_2M–plasmin complexes in a mouse model differs substantially from that of α_2M–trypsin complexes. The clearance of ^{125}I-labeled α_2M–plasmin complexes, formed by reaction of α_2M with a molar excess of plasmin, was considerably slower than that of α_2M–trypsin complexes or α_2M reacted with methylamine. However, upon storage at −20 °C in glycerol for months, the receptor binding properties and in vivo clearance of the α_2M–plasmin complex appear similar to those of the α_2M–trypsin complex (Roche & Pizzo, 1987). In the present study, the properties of binary α_2M–plasmin complexes were examined following 5 min of incubation as well as following several hours of incubation. The properties of these complexes were virtually identical, and thus the mechanism(s) of the subtle changes following storage at −20 °C in glycerol is (are) not known at this time. Examination of the complexes by SDS–PAGE led to the conclusion that the receptor binding properties of the complex appear to be related to the degree of subunit cleavage (Roche & Pizzo, 1987). If this is the case, then it appears that binary α_2M–plasmin complexes, with two subunits cleaved, would differ substantially in both physical and functional properties from ternary complexes, or from α_2M reacted with methylamine.

Alterations occurring primarily in one functional unit in the purified binary α_2M–plasmin complex suggest the possibility that additional proteases can bind to the complex. Studies examining the ability of trypsin to bind to the complex confirm that this is indeed the case and that the α_2M–plasmin complex is capable of binding 0.4 mol of trypsin. These studies are in agreement with those of Jacquot-Armand and Guinand (1976) but appear to differ from those of Gonias and Pizzo (1983b), who examined the ability of the α_2M–plasmin complex to bind trypsin and essentially found no binding. In this case, the initial α_2M–plasmin complex was prepared by incubating a 4.4-fold molar excess of plasmin with the inhibitor for an hour. These conditions should result in complete cleavage of all α_2M subunits (see Figure 5A) and a complete change in the conformation measured by alterations in TNS fluorescence (see Figure 3). Thus, the apparent discrepancy between the data can be accounted for by the fact that the initial conditions utilized to prepare the complex were considerably different and most likely resulted in a different product.

The complexity of the reaction of plasmin with α_2M under conditions of protease excess led us to measure the association rate of plasmin with α_2M under conditions which assured that 1:1 complexes constitute the major product of the reaction. The reaction was carried out under both pseudo-first-order and second-order conditions, and identical results were obtained. In all cases, the concentration of α_2M was held in excess to minimize formation of ternary complexes and to minimize the possibility of nonproductive proteolysis. The reaction rate between α_2M and plasmin is considerably slower than that reported for the reaction of plasmin with α_2-plasmin inhibitor (see Table II). However, unlike the reaction of plasmin with this inhibitor, the reaction rate of plasmin with α_2M is largely unaffected by binding of ligands at the plasmin lysine binding sites. This has been convincingly demonstrated by determining that the association rate of plasmin with this inhibitor is unaltered in the presence of 1 mM ϵ-ACA, and by the fact that the association rate of Val_{442}-plasmin, a derivative lacking the high-affinity lysine binding sites, is virtually identical with that obtained for Lys_{77}-plasmin. From the association rates of plasmin with these two inhibitors and from the plasma concentrations of each inhibitor, the in vivo half-life of plasmin for each inhibitor can be estimated (Bieth, 1980). The results of this calculation, summarized in Table II, demonstrate that, in the absence of any antifibrinolytic agents, the in vivo half-life for plasmin reacting with α_2-plasmin inhibitor is 30–40 ms. However, under conditions where the high-affinity lysine binding sites of plasmin are occupied by antifibrinolytic agents, the in vivo half-life of plasmin reacting with α_2-plasmin inhibitor is increased to 3.0–4.3 s, which is approximately the same half-life estimated for the reaction of plasmin with α_2M, and thus in the presence of antifibrinolytic agents α_2M can effectively compete with α_2-plasmin inhibitor as an important in vivo plasmin inhibitor.

Table II: In Vivo Half-Life of the Reaction of Plasmin with α_2-Plasmin Inhibitor or α_2M

inhibitor	k_a (M^{-1} s^{-1})	I (μM)	$t_{1/2}$ (s)
α_2M	1.34×10^5	2.2–5.8	1.3–2.4
α_2M + 1 mM ϵ-ACA	1.26×10^5	2.2–5.8	1.4–3.6
α_2M + 12 μM HRG	0.31×10^5	2.2–5.8	5.5–14.6
α_2-PI	3.80×10^7 [a]	0.7–1.0	0.03–0.04
α_2-PI + 1 mM ϵ-ACA	3.30×10^5 [b]	0.7–1.0	3.0–4.3

[a] From Wiman and Collen (1978b). [b] From Petersen and Clemmensen (1981).

The role of the plasmin(ogen) lysine binding sites in modulating activity in the fibrinolytic pathway has been the focus of several investigations. Human Glu-plasminogen contains one strong (K_d = 9 μM) and four to five weak (K_d = 5 mM) binding sites for ϵ-ACA (Markus et al., 1978). A model has been proposed in which regulation of fibrinolysis is mediated through the specific interaction of plasmin(ogen) with fibrin, plasminogen activators, and α_2-plasmin inhibitor (Wiman & Collen, 1978a). These specific interactions appear to be mediated by the lysine binding sites located on the heavy chain of the plasmin(ogen) molecule. Saturation of the high-affinity lysine binding sites inhibits the binding of plasmin(ogen) to fibrin (Thorsen, 1975; Wiman & Wallen, 1977) and decreases the rate of inhibition of plasmin by α_2-plasmin inhibitor by 2 orders of magnitude (Petersen & Clemmensen, 1981), thereby altering the molecules that are responsible for the inhibition of plasmin.

The fact that HRG has only a slight effect on the association rate of plasmin with α_2M is interesting. It should be pointed out that it is not known at this time if the HRG–plasmin complex can react with α_2M, or if dissociation of plasmin from the complex is required in order for the reaction to occur. Interesting studies examining the interaction of plasmin with α_2M in the presence and absence of monoclonal antibodies specific for the heavy chain of plasmin(ogen) found that one antibody, when preincubated with plasmin, had only a slight effect on the association rate of the enzyme with α_2M. Two other antibodies appeared to decrease the association rate by approximately 4-fold. These studies found that the heavy chain

of plasmin in the α_2M–plasmin complex is accessible to the antibodies (Cummings & Castellino, 1984). Thus, binding of large molecules to the plasmin heavy chain, which contains the lysine binding regions, seems to only have a moderate effect on the association rate and certainly does not prevent complex formation with α_2M or alter the stoichiometry of plasmin binding with α_2M.

The findings in the present studies that antifibrinolytic agents, including HRG, do not greatly affect the inhibition of plasmin by α_2M offer an explanation for the observation of Harpel (1981) that, under certain conditions, a considerable amount of plasmin formed in plasma associates with α_2M rather than exclusively with α_2-plasmin inhibitor. Thus one function of HRG may be to regulate the distribution of plasmin between various plasma inhibitors. The plasma concentration of HRG has been reported to be approximately 1.8 μM (Lijnen et al., 1980), with a dissociation constant for plasmin(ogen) around 0.9 μM. From these values, it can be estimated that approximately 50% of circulating plasminogen is in complex with this protein. Factors that influence the interaction of HRG with plasmin(ogen) remain to be determined, and more work is required to delineate the role of this molecule in regulating the inhibition of plasmin activity by these two inhibitors as well as to evaluate any potential in vivo significance of α_2M–plasmin complexes.

Registry No. ϵ-ACA, 60-32-2; plasmin, 9001-90-5.

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