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Reaction of Human α_2-Macroglobulin Half-Molecules with Plasmin as a Probe of Protease Binding Site Structure†

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ABSTRACT: Human α_2-macroglobulin (α_2M) half-molecules were prepared by limited reduction and alkylation of the native protein. Reaction with plasmin resulted in nearly quantitative cleavage of the half-molecule M_r ~180 000 subunits into M_r ~90 000 fragments. Subunit cleavage was significantly less complete when plasmin was reacted with α_2M whole molecules. The plasmin and trypsin binding capacities of the two forms of α_2M were compared by using radioiodinated proteases. α_2M half-molecules bound an equivalent number of moles of plasmin or trypsin. Native unreduced α_2M bound only half as much plasmin as trypsin. These data are consistent with the hypothesis that the two protease binding sites are adjacent in native α_2M. α_2M half-molecule–plasmin complexes reassociated less readily than half-molecule–trypsin complexes, supporting this interpretation. The frequency of covalent bond formation between plasmin and α_2M was considerably higher than that previously observed with other proteases. Approximately 80–90% of the plasmin that reacted with α_2M whole molecules or half-molecules became covalently bound. The reactivities of purified α_2M–plasmin complexes were compared with small and large substrates. Equivalent k_{cat}/K_m values were determined at 22 °C for the hydrolysis of H-D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride by whole molecule–plasmin complex and half-molecule–plasmin complex (40 mM^{-1} s^{-1} and 39 mM^{-1} s^{-1}, respectively, compared with 66 mM^{-1} s^{-1} determined for free plasmin). Complexes of plasmin and the two different forms of α_2M digested fibrinogen at comparable rates (slowly compared with free plasmin); however, the half-molecule–plasmin complex demonstrated increased reactivity with soybean trypsin inhibitor. α_2M half-molecule–plasmin complex cleared rapidly from the circulation of mice, reflecting receptor binding and endocytosis.

The plasma protease inhibitor human α_2-macroglobulin (α_2M) is composed of four equivalent M_r ~180 000 polypeptide chains that are associated into pairs by disulfide bonds and whole molecules by strong noncovalent interactions (Swenson & Howard, 1979a; Harpel, 1973; Hall & Roberts, 1978). Each mole of tetrameric α_2M can bind up to 2 mol of α-chymotrypsin or trypsin (Barrett et al., 1979; Swenson & Howard, 1979a; Pochon et al., 1978), but only 1 mol of plasmin (Ganrot, 1967a; Gonias et al., 1982a; Pochon et al., 1978) or 1 mol of a synthetic α-chymotrypsin dimer (Pochon et al., 1981). This variation in molar binding ratios may be explained by a model of α_2M structure that includes two

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adjacent and equivalent protease binding sites (Pochon et al., 1981; Pochon & Bieth, 1982). A large protease bound to one α_2M binding site could then inhibit reaction of a second protease by imposing steric hindrance.

α_2M irreversibly binds proteases from all four major classes (Barrett & Starkey, 1973). A covalent bond may be formed by reaction of a protease nucleophilic amino acid with one of the inhibitor thiol ester bonds present on each subunit (Salvesen et al., 1981; Sottrup-Jensen et al., 1980). Covalent bond formation, however, is not critical for the stability of the inhibitor–protease complex (Salvesen & Barrett, 1980). The α_2M thiol ester bonds also react with small primary amines to yield free sulfhydryl groups and N^{γ}-alkylglutamine residues (Swenson & Howard, 1979b; Sottrup-Jensen et al., 1980). This reaction is accompanied by an α_2M conformational change that is very similar to the conformational change caused by proteases (Barrett et al., 1979; Gonias et al., 1932b). Both reacted forms of α_2M are bound and endocytosed by cell surface receptors that do not recognize unreacted inhibitor (Imber & Pizzo, 1981; Kaplan et al., 1981; Van Leuven et al., 1979).

Tetrameric α_2M may be converted into a homogeneous population of half-molecules by sequential reaction with dithiothreitol and iodoacetamide under nondenaturing conditions (Gonias & Pizzo, 1983a). The modified inhibitor retains many of the functional properties of whole molecules including the capacity to bind trypsin covalently and noncovalently. It was postulated, therefore, that each half-molecule retains one intact protease binding site with two nonautolyzed thiol ester bonds (Gonias & Pizzo, 1983a). The results presented here demonstrate that the reactions of α_2M half-molecules with endopeptidases are not controlled by steric hindrance, as might be predicted by the model of Pochon et al. (1981), since a bound endopeptidase can no longer interact with an adjacent protease binding site. In addition, it is demonstrated that a greater percentage of reacted plasmin is covalently bound to α_2M half-molecules or whole molecules than previously suggested for the reaction of whole molecules with a number of other proteases (Salvesen & Barrett, 1980). Finally complexes of plasmin with half and whole α_2M are purified and compared in small substrate kinetics experiments, fibrinogenolysis studies, and in vivo receptor binding and endocytosis experiments.

Experimental Procedures

Reagents. The peptide substrate H-D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride was obtained from Kabi Diagnostica. The sources for other reagents are listed elsewhere (Gonias & Pizzo, 1983a).

Proteins. α_2M was purified from human plasma as described by Kurecki et al. (1979) and modified by Imber & Pizzo (1981). The reaction conditions for preparing α_2M half-molecules are described in detail elsewhere (Gonias & Pizzo, 1983a). The absorption coefficients ($A^{1\%,1cm}_{280nm}$) of α_2M whole molecules and half-molecules are 8.93 and 9.38, respectively (Hall & Roberts, 1978; Gonias & Pizzo, 1983a). Streptokinase was purified from Kabikinase (Kabi Diagnostica) according to the procedure of Castellino et al. (1976). Plasminogen was purified from human plasma by affinity chromatography on lysine–Sepharose as described by Deutsch & Mertz (1970) and modified by Brockway & Castellino (1972). Affinity chromatography variant two was utilized in all experiments. Protein concentrations in purified preparations of plasminogen and streptokinase were determined by absorption at λ 280 nm as previously described (Gonias et al., 1982a). Plasminogen was activated with streptokinase according to the method of Gonias et al. (1982a) except that the molar excess of plasminogen was reduced to 200. Trypsin was purchased from Worthington Biochemicals. Active site titration was used to determine the concentration of active enzyme in plasmin and trypsin preparations (Chase & Shaw, 1967, 1969). Soybean trypsin inhibitor was purchased from Sigma. Fibrinogen, L grade, was purchased from Kabi Diagnostica and resolved from contaminating plasminogen by passage down lysine–Sepharose. The fibrinogen was then dialyzed extensively against 0.04 M sodium phosphate and 0.15 M NaCl, pH 7.4.

Protein Radioiodination. α_2M and plasminogen were radioiodinated as previously described (Gonias & Pizzo, 1983a). Specific activities, expressed in the units cpm per picomole are listed below.

Polyacrylamide Gel Electrophoresis. Denatured proteins were electrophoresed in an ammediol buffered gel system with sodium dodecyl sulfate ($NaDodSO_4$) in the upper reservoir and sample buffer as described by Wyckoff et al., (1977). Samples containing plasmin were allowed to react with 0.08 mM *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride prior to denaturation, unless otherwise specified.

Radioiodinated Plasmin Binding Experiments. Equivalent weights of α_2M half-molecules (25.0 pmol) or α_2M whole molecules (12.5 pmol) were reacted with 55.0 pmol of active radioiodinated plasmin (6×10^4 cpm/pmol) for 60 min at 22 °C in 0.02 M sodium phosphate and 0.1 M NaCl, pH 7.4. The reaction mixture was then chromatographed on an Utrogel AcA-22 column (28 × 0.8 cm) at 2–3 mL/h, in the same buffer. The radioactivity contained within fraction recovered from the column (350 μL) was measured in a Scientific Products AW 14-120 γ counter. Binding of plasmin to α_2M was calculated based on the radioactivity eluting in the first peak (high molecular weight) as previously described (Gonias & Pizzo, 1983a) and compared with the results of similar binding experiments performed with trypsin and α_2M, presented elsewhere (Gonias et al., 1982b; Gonias & Pizzo, 1983a).

Binding experiments were also performed in which radioiodinated plasmin was reacted with α_2M half-molecules or whole molecules for 60 min as described above. Soybean trypsin inhibitor, at 5 times the active plasmin concentration, was then added to each reaction mixture for an additional 20 min prior to chromatography.

Covalent binding of radioiodinated plasmin to α_2M half-molecules or whole molecules was studied with denaturing gel electrophoresis as described elsewhere (Harpel & Hayes, 1979; Salvesen & Barrett, 1980; Gonias & Pizzo, 1983a) and modified as follows. Radioiodinated plasmin (40 pmol) (3.4×10^4 cpm/pmol) was reacted with equivalent weights of α_2M half-molecules or whole molecules (9 pmol of whole molecules and 18 pmol of half-molecules) for 60 min at 22 °C. The two samples were denatured in the absence of reductant and electrophoresed on 5% gels that were stained with Coomassie Brilliant Blue R (Sigma) and scanned with a densitometer as previously described (Gonias & Pizzo, 1981). Each gel was then sliced into 20 equivalent sections that were counted for radioactivity in a γ counter. As a control, a third sample containing 40 pmol of plasmin and no α_2M was subjected to the same procedure. The amount of plasmin covalently bound to α_2M was calculated from the radioactivity recovered in gel sections of lower R_f (higher molecular weight) than unreacted plasmin. Recovery of radioiodinated protein in chromatography and electrophoresis binding experiments was between 90 and 100%.

Reassociation Experiments. α_2M half-molecules reassociated to form whole molecules (four subunits) after reaction

with trypsin or methylamine (Gonias & Pizzo, 1983a). The association properties of half-molecules after reaction with plasmin were studied with molecular exclusion chromatography on an Utrogel AcA-22 column (30 × 1.4 cm) equilibrated with 0.02 M sodium phosphate and 0.1 M NaCl, pH 7.4. In each experiment, 400–550 μg of the protein of interest was cochromatographed with between 0.1 and 0.5 μg of radioiodinated α_2M whole molecule–trypsin complex [(2.5–5.0) × 10^5 cpm/pmol] as a calibration marker. Recovered elution fractions (400 μL) were measured for absorbance at λ 280 nm and radioactivity in a γ counter.

Steady-State Kinetics. The rate of hydrolysis of the chromogenic peptide substrate H-D-Val-leu-Lys-*p*-nitroanilide dihydrochloride by equivalent concentrations of free active plasmin and plasmin complexed with either half-molecules or whole molecules of α_2M (15.5 nM) was studied at 22 °C in 0.02 M sodium phosphate and 0.1M NaCl, pH 7.4. The α_2M–plasmin complexes were formed by utilizing the reaction conditions described for the plasmin binding experiments and purified by chromatography on Ultrogel AcA-22. Small aliquots of radioiodinated α_2M were included in the reaction mixture so that the concentration of complex could be accurately determined after chromatography. Substrate concentration was varied between 0.05 and 1.8 mM. Hydrolysis was monitored at λ 405 nm utilizing a molar extinction coefficient of 9950 for *p*-nitroaniline (Lottenberg & Jackson, 1983). Steady-state kinetic parameters were determined for α_2M–plasmin complexes by using the binding ratio data listed under Results (Table I). All experiments were performed at least in duplicate and the results averaged.

Soybean Trypsin Inhibitor Resistant Amidase Activity Assay. Identical amounts of active plasmin (13 pmol) were reacted with different concentrations of α_2M half-molecules or whole molecules for 60 min at 22 °C. Soybean trypsin inhibitor at 6 times the concentration of plasmin was then added to each incubation mixture for an additional 30 min. Hydrolysis of the substrate H-D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride (0.6 mM) was monitored as described above and compared with an equivalent sample of plasmin that was incubated for 90 min without inhibitors. In control experiments, soybean trypsin inhibitor inhibited 96–100% of the amidase activity of free plasmin. Unreacted α_2M preparations demonstrated less than 0.03% of the activity associated with an equivalent weight of plasmin. This procedure was derived, in part, from the methods presented by Ganrot (1967b).

Fibrinogenolysis Experiments. Complexes of plasmin and α_2M half-molecules or whole molecules were purified as described for the small substrate kinetics experiments. Dissociation of protease was below detectable limits when complex was passed down the Ultrogel AcA-22 column a second time. Equal concentrations (0.09 μM) of free plasmin, α_2M whole molecule–plasmin complex, and α_2M half-molecule–plasmin complex were incubated separately with fibrinogen (1 mg/mL) in 0.02 M sodium phosphate and 0.1 M NaCl, pH 7.4 at 37 °C. At the designated time points, 14-μL aliquots were removed, incubated with *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (30 μM) for 4 min at room temperature, and frozen. Upon completion of a study, all of the frozen samples were thawed simultaneously and denatured in 1% $NaDodSO_4$ (no reductant) at 70 °C. Denaturing gel electrophoresis was then performed as described above. In control experiments, fibrinogen that was incubated in 0.02M sodium phosphate and 0.1 M NaCl, pH 7.4, for 20 h at 37 °C in the absence of protease remained totally undigested as determined with gel electrophoresis.

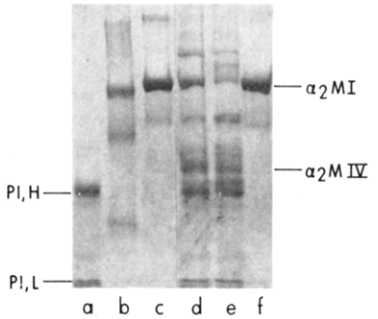

FIGURE 1: Polyacrylamide gel electrophoresis of α_2M whole molecules and half-molecules before and after reaction with plasmin. All samples were simultaneously reduced and denatured prior to electrophoresis except sample b that was denatured in the absence of reductant. (a) Plasmin, (b and c) α_2M half-molecules, unreacted, (d) α_2M whole molecules reacted with plasmin, (e) α_2M half-molecules reacted with plasmin, and (f) α_2M whole molecules, unreacted. An equivalent quantity (7 μg) of α_2M was electrophoresed in lanes b–f (5% polyacrylamide slab). The labeled bands include the following: α_2M I, the M_r ~180000 α_2M subunit; α_2M IV, the M_r ~90000 α_2M subunit cleavage product resulting from reaction with protease; Pl, H, the plasmin heavy chain; Pl, L, the plasmin light chain.

In Vivo Plasma Clearance Studies. In vivo receptor binding and endocytosis studies of radioiodinated α_2M derivatives were performed in CD-1 female mice (Charles River). Human α_2M and mouse α_2M, when reacted with protease, clear equivalently in mice reflecting interaction with receptors accessible to the vascular space (Gonias et al., 1983). Detailed descriptions of the procedure (Imber & Pizzo, 1981) and the theory of these experiments (Gonias & Pizzo, 1983b) are presented elsewhere.

Results

α_2M Subunit Cleavage by Plasmin. α_2M half-molecules were electrophoresed after denaturation in the presence and absence of dithiothreitol (Figure 1). The M_r ~180000 single subunit band was the major component of both preparations. Simultaneous denaturation and reduction yielded more intensely staining bands, reflecting the increased capacity of the completely reduced molecules to bind dye. Faint high R_f bands resulted from the autolysis of some thiol ester bonds exposed to denaturant, as described by Harpel et al. (1979).

α_2M whole molecules were reacted for 60 min at room temperature with a 5-fold molar excess of active plasmin. Cleavage of the α_2M subunits into M_r ~90000 fragments, which occurs quantitatively during reaction with some proteases such as trypsin (Harpel, 1973; Gonias et al., 1982a), was incomplete for the reaction with plasmin. This result has been shown to remain unchanged after incubation of α_2M with higher concentrations of plasmin and at elevated temperature (37 °C) (Gonias et al., 1982a).

α_2M half-molecules were reacted with plasmin under conditions equivalent to those described above. The partially reduced inhibitor subunits appeared to be cleaved only once, at the same site as the unreduced inhibitor. The reaction was, however, substantially more complete. Only a faint band electrophoresing at the position of intact single subunits remained.

Comparison of the Plasmin and Trypsin Binding Capacities of α_2M. Unreduced α_2M bound 1.10 mol of radioiodinated plasmin per mol of inhibitor when the two proteins were reacted under the conditions described under Experimental Procedures and chromatographed on Ultrogel AcA-22. This result is in good agreement with previous studies (Ganrot, 1967a; Gonias et al., 1982a; Pochon et al., 1978). Incubation of α_2M–plasmin with soybean trypsin inhibitor prior to chromatography resulted in the dissociation of little or none

Table I: Binding of Radioiodinated Plasmin to α_2M Half-Molecules and α_2M Whole Molecules

type of binding	mol of plasmin bound per: mol of whole molecules	mol of plasmin bound per: 2 mol of half-molecules	ratio of plasmin bound/trypsin bound under equivalent conditions[a]: whole molecules	ratio of plasmin bound/trypsin bound under equivalent conditions[a]: half-molecules
total binding	1.10	1.14	0.45	0.85
total binding after reaction with soybean trypsin inhibitor	1.07	1.04	0.51	1.04
covalent binding	0.87	0.95	0.82	1.13

[a] Data concerning the reaction of α_2M with trypsin are extracted from the following references: Gonias & Pizzo (1983a); Gonias et al. (1982b).

of the complexed plasmin. Table I presents comparisons of the plasmin and trypsin binding capacities of α_2M. α_2M whole molecules bound only about half as much plasmin as trypsin when studied with equivalent experiments.

α_2M half-molecules bound 0.57 and 0.52 mol of plasmin/mol when studied with and without subsequent incubation with soybean trypsin inhibitor. These values are comparable to those obtained for α_2M whole molecules when they are corrected so that equivalent weights of each form of the inhibitor are compared. Trypsin binding to half-molecules, when studied with chromatography, was somewhat decreased compared to the unreduced inhibitor (Gonias & Pizzo, 1983a). This decrease was attributed to reversible dissociation of noncovalently bound trypsin that occurs during chromatography of half-molecule complexes but not whole molecule complexes. The values presented for plasmin binding to half-molecules are most likely minimum estimates for the same reason. In contrast with the unreduced inhibitor, the α_2M half-molecule binding capacities for the large protease, plasmin, and the smaller protease, trypsin, were equivalent (Table I).

α_2M half-molecules and whole molecules were reacted with radioiodinated plasmin, denatured in the absence of reductant, and subjected to gel electrophoresis to study covalent binding. Nonreducing conditions were chosen so that both the heavy and light chains of plasmin, which are disulfide bonded in the active protease (Robbins et al., 1967), would remain associated with the inhibitor in denaturant, even if only one of the two polypeptide chains were covalently bound to an α_2M subunit. As a control, unreacted plasmin was electrophoresed alone (Figure 2). A single symmetric radioactivity and dye binding peak was observed with the isolated protease. Two additional high molecular weight bands were observed in the stained gel, when plasmin was reacted with α_2M whole molecules. Radioactive protease was recovered in gel sections that included both of these bands; however, the majority of the plasmin appeared to be associated with the lowest R_f band. Covalent binding of plasmin to native α_2M (whole molecules) was 0.87 mol/mol. Approximately 80% of the plasmin that was trapped by α_2M was covalently bound as well.

The reaction of plasmin with α_2M half-molecules resulted in a somewhat more complicated pattern of electrophoresis bands, reflecting the absence of inhibitor intersubunit covalent bonds. Radioactivity was recovered in seven low R_f sections distributed around sections one and four. Covalent binding of plasmin to the α_2M half-molecules was 0.47 mol/mol. This value represents greater than 80% of the plasmin binding to

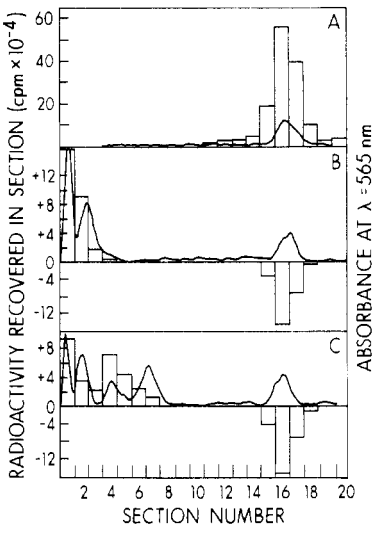

FIGURE 2: Covalent bond formation during the reaction of radioiodinated plasmin with α_2M whole molecules and half-molecules. Equivalent quantities of plasmin were electrophoresed before reaction in panel A, after reaction with α_2M whole molecules in panel B, and after reaction with α_2M half-molecules in panel C. Densitometry scans of the stained gels are reproduced with a solid line. The radioactivity recovered in each gel section is shown in panel A with a bar graph. The bar graphs in panels B and C show the amount of radioactivity recovered in each gel section relative to the equivalent section in panel A. A negative bar in panel B or C reflects the presence of less radioactivity in the given section compared with that in panel A, whereas a positive bar reflects the presence of more radioactivity.

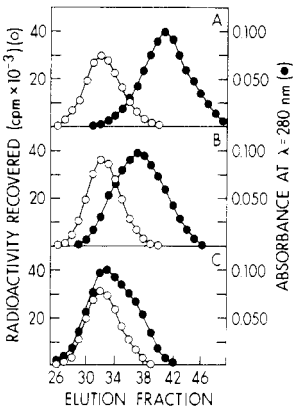

FIGURE 3: Molecular exclusion chromatography of α_2M half-molecules. (A) Unreacted half-molecules; (B) half-molecules reacted with a 2-fold molar excess of plasmin for 2 h; (C) half-molecules reacted with a 2-fold molar excess of trypsin for 2 h. A small amount of radioiodinated α_2M whole molecule–trypsin complex was cochromatographed with each preparation. In panel B, unreacted plasmin eluted in a symmetric peak centered around fractions 52 and 53. In a control experiment not shown, 500 μg of α_2M whole molecules were reacted with a 4-fold molar excess of plasmin for 2 h and cochromatographed with radioiodinated whole molecule–trypsin complex. A significant difference between the elution positions of the radioactivity peak and the absorbance peak was not observed.

half-molecules detected with chromatography experiments and 110% of covalent binding capacity demonstrated by unreduced α_2M (comparison of equal weights).

Reassociation of α_2M Half-Molecules after Reaction with Plasmin. α_2M half-molecules reassociate (forming whole molecules of four subunits each) when reacted trypsin or methylamine (Gonias & Pizzo, 1983a). Both reactions are accompanied by a significant and equivalent increase in the Stokes radius of the inhibitor. Figure 3 compares the Stokes radii of α_2M whole molecules and half-molecules that were reacted with plasmin or trypsin. Reaction of half-molecules with plasmin yielded a product of increased radius as judged

Table II: Steady-State Kinetic Parameters for the Hydrolysis of H-D-Val-Leu-Lys-*p*-nitroanilide Dihydrochloride by Plasmin and α_2M–Plasmin Complexes[a]

protease	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
plasmin	0.18 ± 0.02	11.8 ± 0.4	66
plasmin complexed to α_2M whole molecule	0.21 ± 0.03	8.5 ± 0.4	40
plasmin complexed to α_2M half-molecule	0.25 ± 0.03	9.7 ± 0.3	39

[a] Conditions: at 22 °C in 0.02 M sodium phosphate and 0.1 M NaCl, pH 7.4.

by chromatography on an Ultrogel AcA-22 column. The observed shift was, however, significantly smaller than that observed when half-molecules were reacted with trypsin, suggesting that the larger protease, plasmin, may sterically prevent the extensive reassociation observed for other forms of reacted inhibitor half-molecules. Insufficient data are currently available to determine whether the difference in Stokes radius between α_2M half-molecule–plasmin complex and the unreacted halves results from protrusion of the protease beyond the longest axis of the modified inhibitor or a low level of rapidly reversible reassociation.

Plasmin and α_2M–Plasmin-Catalyzed Hydrolysis of a Tripeptide p-Nitroanilide. The hydrolysis of H-D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride by plasmin was examined under the conditions utilized in experiments with α_2M–plasmin complexes. A Lineweaver–Burk plot of the data was linear within the studied substrate concentration range (data not shown). Steady-state kinetic parameters for the reaction are presented in Table II. A slightly higher k_{cat} and similar K_m were obtained when the experiment was repeated in 0.05 M Tris-HCl and 0.15 M NaCl, pH 7.4. These data are consistent with those presented for the hydrolysis of H-D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride at 25 °C by Christensen & Ipsen (1979).

Plasmin that was purified in complex with α_2M whole molecules or α_2M half-molecules retained significant catalytic activity in the hydrolysis of the substrate H-D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride (Table II). Linear Lineweaver–Burk plots were obtained with both complexes. The k_{cat} determined for plasmin bound to unreduced α_2M was substantially higher than a previously reported value determined at 37 °C with the same substrate (Gyzander & Teger-Nilsson, 1980). The k_{cat}/K_m values determined for plasmin complexed to inhibitor whole molecules and plasmin complexed to inhibitor half-molecules were equivalent despite the slight differences noted in K_m and k_{cat}. Both protease–α_2M complexes retained greater than 70% of the activity demonstrated by free plasmin when saturated with substrate.

α_2M–Plasmin-Catalyzed Small Substrate Hydrolysis in the Presence of Soybean Trypsin Inhibitor. α_2M whole molecules and α_2M half-molecules protected the active site of bound plasmin from inhibition by soybean trypsin inhibitor so that significant substrate hydrolysis was observed (Figure 4). Plasmin incubated with whole molecules retained a maximum of 75% of the activity of the control preparation of free protease, while plasmin incubated with half-molecules retained nearly 50%. These data were analyzed in terms of three factors: (1) the activity of the control preparation of plasmin, (2) the kinetic parameters in Table II, and (3) interactions of α_2M–plasmin complex with soybean trypsin inhibitor. The control preparation of free plasmin lost approximately 10–15%

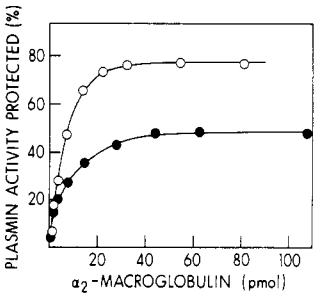

FIGURE 4: Amidolytic activity of plasmin bound to α_2M in the presence of soybean trypsin inhibitor. A constant concentration of plasmin was reacted with varied concentrations of α_2M whole molecules (○) or α_2M half-molecules (●). Soybean trypsin inhibitor was added to each incubation mixture, and small substrate hydrolysis was then measured. Reaction conditions are provided under Experimental Procedures. Activities were determined relative to a control preparation of plasmin that was not reacted with α_2M or soybean trypsin inhibitor.

of its maximal activity during the 90-min incubation period at room temperature. Inactivation of α_2M–plasmin complex occurred much more slowly. These results and the kinetic parameters in Table II adequately account for the activity of plasmin bound to α_2M whole molecules. The lower substrate hydrolysis rate demonstrated by plasmin bound to half-molecules suggests that soybean trypsin inhibitor interacts more extensively with protease bound to the modified inhibitor. This type of interaction has been previously observed with α_2M half-molecule–trypsin complex (Gonias & Pizzo, 1983a).

Fibrinogenolysis of α_2M–Plasmin Complexes. Fibrinogen digestion by plasmin and plasmin bound to α_2M half-molecules and whole molecules was examined with denaturing gel electrophoresis in the absence of reductant as originally described by Pizzo et al. (1972). The results obtained confirm and extend those reported by Harpel & Mosesson (1973). Highly purified unreduced α_2M–plasmin complex degraded fibrinogen slowly but completely so that only the terminal digestion products, fragment D and fragment E, remained at 20 h (Figure 5). This extent of digestion indicates that the highly resistant fibrinogen γ chain was cleaved by the inhibitor–plasmin complex as well as the more susceptible Aα and Bβ chains.

α_2M half-molecule–plasmin complex digested fibrinogen at a rate comparable to that observed with whole molecule–plasmin complex. The slightly more rapid degradation of fragment Y and the presence of fragment D heterogeneity at 20 h may indicate that plasmin retained slightly less restricted fibrinogenolytic activity when bound to half-molecules. These differences were, however, extremely small when compared with those observed between the α_2M–plasmin complexes and the unreacted protease. Free plasmin completely digested fibrinogen so that no intact protein, fragment X, or fragment Y remained within 20 min when studied under the reaction conditions used in the digestion experiments above. Additional samples removed at later time intervals showed only some increase in fragment D heterogeneity, as expected (Pizzo et al., 1972). These experiments suggest that plasmin bound to half-molecules is sterically inhibited in its reaction with large substrates as are proteases that are bound to unreduced α_2M.

In Vivo Receptor Binding and Endocytosis of α_2M–Plasmin Complex. α_2M–protease complexes clear from the circulation of mice rapidly, reflecting binding and endocytosis by receptors that do not recognize the unreacted inhibitor (Imber & Pizzo, 1981; Gonias & Pizzo, 1982a, 1983b). Figure 6 compares the clearance rates of α_2M half-molecules and whole molecules before and after reaction with plasmin. Both forms of inhibitor–plasmin complex cleared at accelerated rates when

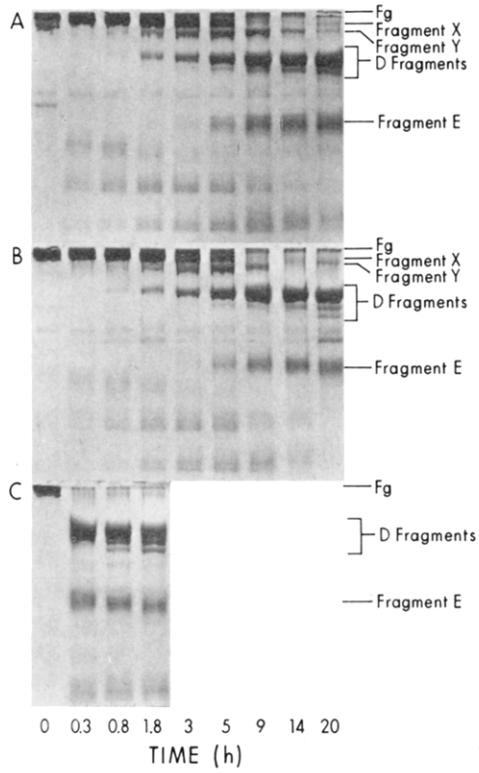

FIGURE 5: Fibrinogenolysis by plasmin bound to α_2M whole molecules and half-molecules. Complexes were purified as described in the text. Panel A shows the digestion of fibrinogen by α_2M whole molecule–plasmin, panel B, α_2M half-molecule–plasmin, and panel C, free plasmin. Electrophoresis was conducted on a 7.5% polyacrylamide slab. Fg, fibrinogen (undigested).

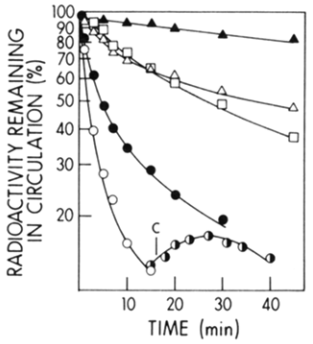

FIGURE 6: In vivo plasma elimination experiments comparing α_2M whole molecules and half-molecules after reaction with plasmin. In each experiment, 0.5–2.0 μg or radioiodinated α_2M (6×10^5 cpm/pmol) was injected into the lateral tail vein of a CD-1 female mouse. At varied time points, 25-μL blood samples were removed from the retroorbital venous plexus and measured for radioactivity in a γ counter. The radioactivity remaining in the circulation was calculated as a percentage relative to an initial blood sample drawn approximately 5–10 s after injection. The studies shown include: unreacted α_2M half-molecules (△), unreacted α_2M whole molecules (▲), half-molecules reacted with a 2-fold molar excess of plasmin for 60 min (○), whole molecules reacted with a 4-fold molar excess of plasmin (●), and half-molecules reacted with plasmin and injected in the presence of a 750-fold molar excess of nonradioactive α_2M whole molecule–trypsin complex (□). In a final experiment, α_2M half-molecule–plasmin was injected into the circulation and allowed to clear for 16 min. At the point labeled C, a 750-fold molar excess of α_2M whole molecule–trypsin complex was administered into the circulation with a second injection (◑).

compared with the unreacted inhibitors. Significant inhibition of clearance was observed when radioiodinated α_2M half-molecule–plasmin complex was injected in the presence of a 750-fold molar excess of nonradioactive α_2M whole molecule–trypsin complex. This result demonstrates that the α_2M receptor is responsible for the rapid plasma elimination of the half-molecule–plasmin complex. A second form of competition experiment was performed in which a large molar excess of α_2M–trypsin complex was injected after the radioiodinated half-molecule–plasmin complex was allowed to clear for 16 min. This type of study distinguishes between radioligand that is endocytosed and radioligand that is reversibly bound to cell surfaces (Gonias & Pizzo, 1983a,b). The competing ligand displaced back into the circulation only a small percentage of the half-molecule–plasmin complex that had apparently cleared, suggesting that endocytosis as well as receptor binding had occurred.

Discussion

The four identical subunits of native α_2M are arranged to form two distinct protease binding sites (Pochon et al., 1978; Swenson & Howard, 1979a). A number of studies suggest that these protease binding sites are equivalent, noninteracting (Pochon & Bieth, 1982; Gonias & Pizzo, 1983a), adjacent (Pochon et al., 1981), and located on the protein surface (Gonias et al., 1982b; Bieth et al., 1981). The inability of α_2M to bind 2 mol of a large protease such as plasmin can be explained by steric hindrance caused by the extension of one protease into the second binding site (Pochon et al., 1981; Ganrot, 1967a). This hypothesis is supported by results presented in this paper suggesting that the reaction of plasmin with half-molecules is not controlled by steric hindrance. The consistent results include the following: (1) Each mole of α_2M half-molecules bound an equivalent number of moles of plasmin or trypsin. As expected, unreduced α_2M bound only half as much plasmin as it bound trypsin. (2) The cleavage of the α_2M half-molecule subunits into M_r ~90000 fragments was nearly complete after reaction with plasmin. Studies of native α_2M have shown that each reacting molecule of endoprotease cleaves two of the four inhibitor subunits (Pochon et al., 1981; Gonias et al., 1982a; Sottrup-Jensen et al., 1981). The reaction of α_2M whole molecules with plasmin resulted in significantly less complete subunit cleavage. (3) α_2M half-molecules reassociated extensively to form whole molecules after reaction with trypsin or methylamine (Gonias & Pizzo, 1983a). Reassociation of half-molecules after reaction with plasmin was substantially less complete. It is postulated that the same steric considerations that prevent an α_2M whole molecule from binding two molecules of plasmin also inhibit reassociation of two half-molecule–plasmin complexes.

Recently, Howell et al. (1983) proposed a somewhat different explanation for why α_2M binds two molecules of certain proteases and only one molecule of others. Saturation of the second protease binding site was postulated to reflect the ability of the protease to bind to the inhibitor and cleave the second pair of α_2M subunits before conformational change occurred resulting in the formation of an irreversible 1:1 complex. The results outlined above, supporting the model of Pochon et al. (1981), are also consistent with this second model. A number of laboratories have reported, however, that 1:1 complexes of α_2M with plasmin or α-chymotrypsin dimer retain the capacity to bind small proteases such as trypsin or α-chymotrypsin monomer (Ganrot, 1967a; Pochon et al., 1981; Jacquat-Armand & Guinand, 1976). These results are difficult to reconcile with a model of α_2M–protease binding stoichiometry based on reaction rate alone. It is important to note, however, that the models of Pochon et al. (1981) and Howell et al. (1983) are not mutually exclusive.

The frequency of covalent bond formation between α_2M and plasmin was approximately 80% when determined with the procedure described in this paper. Salvesen & Barrett (1980)

previously reported a lower value (54%) for the same reaction. The difference between the two values reflects the exposure of α_2M–plasmin to reductant during denaturation prior to electrophoresis in the studies presented by Salvesen & Barrett (1980). These conditions would be expected to result in the dissociation of either the plasmin heavy chain or light chain, if one and not the other was covalently bound to an inhibitor subunit. The covalent binding frequency reported in this paper for plasmin and α_2M is also considerably higher than that reported for the reaction of α_2M with a number of proteases that contain single polypeptide chains (Salvesen & Barrett, 1980). This result and other unpublished observations from this laboratory may indicate that protease size is an important factor in predicting covalent bond formation frequency, as is lysine content (Salvesen et al., 1981).

Hydrolysis of the substrate H-D-Val-leu-Lys-*p*-nitroanilide dihydrochloride by free plasmin and plasmin complexed with the two forms of α_2M was studied under a single set of reaction conditions (temperature, pH, and ionic strength). The k_{cat}/K_m values determined for inhibitor-bound forms of plasmin were approximately 60% of the value determined for free plasmin. This result most likely reflects a decrease in the acylation rate of plasmin bound to α_2M. The kinetic parameters determined for plasmin complexed to α_2M whole molecules and plasmin complexed to α_2M half-molecules were very similar. The half-molecule complex may have been deacylated at a slightly faster rate as indicated by the slightly higher k_{cat} and K_m values. Rinderknecht et al. (1975) reported that the optimal conditions for the hydrolysis of a small synthetic substrate are different for free trypsin and α_2M–trypsin complex. Similarly, the kinetic parameters presented here for plasmin and α_2M–plasmin may vary to different extents as a function of the reaction conditions. Gyzander & Teger-Nilsson (1980) reported a k_{cat} of 0.6 s^{-1} for the α_2M–plasmin catalyzed hydrolysis of H-D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride at 37 °C (approximately 7% of the value presented here). It is unlikely that this discrepancy reflects the use of different reaction buffers.

Native α_2M forms complexes with proteases that react with soybean trypsin inhibitor at extremely slow rates (Bieth et al., 1981; Ganrot, 1967b). α_2M half-molecule–protease complexes react with the second inhibitor more readily. These results are consistent with greater exposure of the enzyme active site in the half-molecule complex. It was therefore, somewhat surprising that the fibrinogenolytic activity of plasmin complexed with α_2M half-molecules was inhibited to an extent comparable with that observed of plasmin bound to unmodified α_2M. Apparently, the steric factors contributing to the inhibition of fibrinogenolysis by plasmin complexed to α_2M are somewhat different from those responsible for restricting reactivity with soybean trypsin inhibitor.

Harpel & Mosesson (1973) observed digestion of fibrinogen Aα and Bβ chains by α_2M–plasmin in the presence and absence of pancreatic trypsin inhibitor. These studies indicate that the fibrinogenolytic activity associated with the inhibitor–protease complex is not attributable to a small contaminant of unbound plasmin. The α_2M–plasmin-catalyzed digestion of fibrinogen γ chains observed here and not by Harpel & Mosesson (1973), under similar conditions, probably reflects the use of a more rapid procedure for isolating higher concentrations of active complex.

Complexes of unreduced α_2M and plasmin consistently clear from the circulation of mice at a slightly slower rate than α_2M–trypsin or α_2M–methylamine (Gonias et al., 1982a; Gonias & Pizzo, 1983b). One possible explanation for this result is that the large molecule of bound plasmin interferes, to some small extent, with the interaction between the α_2M receptor and the receptor recognition site (Gonias et al., 1982a). If this explanation were correct, similar interference might be expected to retard the clearance of half-molecule–plasmin complex. The data presented in Figure 6 do not support this hypothesis. The α_2M half-molecule–plasmin complex cleared at a faster rate than either half-molecule–trypsin complex or unreduced α_2M–plasmin complex. A second model currently under investigation considers the slower clearance rate of unreduced α_2M–plasmin the result of incomplete subunit cleavage. The data presented in this paper are consistent with this model.

Registry No. H-D-Val-Leu-Lys-*p*-nitroanilide, 63589-93-5; plasmin, 9001-90-5; trypsin, 9002-07-7.

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Isolation and Characterization of Pepsin-Solubilized Human Basement Membrane (Type IV) Collagen Peptides†

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ABSTRACT: Native type IV collagen was isolated from human placental tissue by pepsin digestion, fractional salt precipitation, reduction and alkylation, a second pepsin digestion, and chromatography on diethylaminoethyl- and carboxymethylcellulose. After denaturation, 10 distinct peptides were isolated from this material by molecular sieve, ion-exchange, and high-performance liquid chromatography. All of the peptides were found to have amino acid compositions characteristic of type IV collagen. Analysis of the eight major peptides by amino-terminal amino acid sequencing and by cyanogen bromide and tryptic peptide mapping has revealed the manner in which they are derived from type IV collagen. Pepsin liberates two large peptides by attacking non-triple-helical regions, one derived from the α1(IV) chain (F2, M_r 90 000) and one derived from the α2(IV) chain (F3, M_r 75 000). The α1(IV)-derived F2 peptide is also represented in the pepsin digest by amino-terminal and carboxy-terminal subfragments [F4c (M_r 41 000) and F4a (M_r 60 000)], as is the α2(IV)-derived F3 peptide [F5 (M_r 28 000) and F4b (M_r 50 000), respectively]. These findings indicate that the molecular regions from which the larger peptides are derived in themselves contain pepsin-sensitive (non-triple-helical) domains. In addition, several of the peptides examined were found to be present in two slightly different forms, suggesting that closely adjacent pepsin-sensitive sites often exist within the type IV collagen molecules. The methods outlined here provide a reliable means by which identifiable type IV collagen peptides can be isolated. Furthermore, the above conclusions and the data from which they have been drawn provide a basis from which the previously described type IV collagen peptides can be more clearly identified and related to a common structure of origin.

Basement membranes are sheetlike extracellular matrices which support and compartmentalize soft tissue structures (Vracko, 1974) and also serve as selective barriers to permeability (Caufield & Farquhar, 1978). Chemical and immunological studies of basement membranes have shown the presence of the glycoproteins laminin (Timpl et al., 1979a) and fibronectin (Stenman & Vaheri, 1978), a heparin sulfate containing proteoglycan (Hassell et al., 1980), and a major collagenous component known as type IV collagen. Type IV collagen is chemically and structurally distinct from the interstitial collagens (Kefalides, 1973) and is believed to be the primary structural element of basement membranes (Bornstein & Sage, 1980).

Recent studies on the type IV collagen secreted by cell and tissue cultures have shown that two distinct polypeptides are produced, pro-α1(IV) and pro-α2(IV), with molecular weights of 185 000 and 175 000, respectively (Crouch & Bornstein, 1979; Alitalo et al., 1980; Crouch et al., 1980; Fessler & Fessler, 1980; Tryggvason et al., 1980). Although processing of these biosynthetic products to smaller chains has not been observed under culture conditions, type IV collagen polypeptides with molecular weights of 160 000 and 140 000 have been extracted from bovine lens capsules (Gay & Miller, 1979) and from the matrix of the EHS murine tumor when grown

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