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Binding and Endocytosis of α_2-Macroglobulin–Plasmin Complexes†

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ABSTRACT: The clearance of ^{125}I-labeled α_2-macroglobulin–plasmin complexes (^{125}I-α_2M–PM) from mouse circulation is slower than that of ^{125}I-labeled α_2M–methylamine complexes (^{125}I-α_2M–CH_3NH_2). In addition, clearance of ^{125}I-α_2M–PM is biphasic, but that of ^{125}I-α_2M–CH_3NH_2 follows simple first-order kinetics. Treatment of α_2M–PM with trypsin yields a complex that clears like α_2M–CH_3NH_2. Complexes of α_2M with Val_{442}-plasmin (α_2M–Val_{442}-PM) were prepared; α_2M–Val_{442}-PM has a stoichiometry of 2 mol of Val_{442}-PM to 1 mol of α_2M and also clears like α_2M–CH_3NH_2. In vitro 4 °C binding inhibition studies with mouse peritoneal macrophages show that α_2M–CH_3NH_2, α_2M–PM, trypsin-treated α_2M–PM, and α_2M–Val_{442}-PM bind with the same affinity, apparent K_d = 0.4 nM. The binding isotherms at 4 °C are the same for ^{125}I-α_2M–CH_3NH_2, ^{125}I-α_2M–PM, and ^{125}I-trypsin-treated α_2M–PM in both mouse peritoneal macrophages and 3T3-L1 fibroblasts. The Scatchard plots for the binding isotherms in macrophages were curved; those in 3T3-L1 fibroblasts were linear with an apparent K_d of 0.48 nM and a receptor activity of 140 fmol/mg of cell protein for α_2M–CH_3NH_2, an apparent K_d of 0.29 nM and a receptor activity of 110 fmol/mg of cell protein for α_2M–PM, and an apparent K_d of 0.35 nM and a receptor activity of 210 fmol/mg of cell protein for trypsin-treated α_2M–PM. The time course of uptake and degradation and the concentration dependence of uptake at 37 °C are indistinguishable for ^{125}I-α_2M–PM and ^{125}I-α_2M–CH_3NH_2 in mouse peritoneal macrophages. The uptake of both complexes reaches a steady state after 1–2 h. Maximum cell-associated ligand at steady state is 2.0 pmol/mg of cell protein, and the concentration of half-maximal uptake is 50 nM for both complexes. These results suggest that the slower clearance of α_2M–PM from mouse circulation is not due to altered receptor recognition of the α_2M–PM complex but may be due to α_2M–PM interaction with some factor in mouse blood.

α_2-Macroglobulin (α_2M) is a large (M_r 718 000) plasma glycoprotein which binds to and inhibits endoproteases from each of the four classes serine, cysteine, metallo, and carboxyl [for reviews, see Starkey & Barrett (1977) and Barrett (1981)]. Protease binds to a "bait region" on α_2M (Barrett & Starkey, 1973; Sottrup-Jensen et al., 1981b) and then cleaves the bait region (Mortensen et al., 1981), causing a conformational change (Barrett et al., 1974; Nelles et al., 1980) in α_2M which sterically traps the protease (Barrett & Starkey, 1973) and exposes a reactive thiol ester (Sottrup-Jensen et al., 1981a). The exposed thiol ester is cleaved (Sottrup-Jensen et al., 1980) by nucleophilic attack by the bound protease or other nucleophiles in solution (Sottrup-Jensen et al., 1981c). Primary amines such as methylamine cleave the thiol ester (Sottrup-Jensen et al., 1980) and produce a conformational change without cleavage of the bait region (Steinbuch et al., 1968).

The conformational change and thiol ester cleavage following reaction of α_2M with protease or methylamine expose a receptor recognition site (Ohlsson, 1971a–c; Imber & Pizzo, 1981; Kaplan et al., 1981; Marynen et al., 1981). Once α_2M has reacted with protease or methylamine and becomes the "fast form" of α_2M, it is rapidly cleared from the circulation of dogs, humans, or mice (Ohlsson, 1971a–c; Blatrix et al.,

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1973; Imber & Pizzo, 1981). Cells in culture, including human fibroblasts (Mosher & Vaheri, 1980; Van Leuven et al., 1978, 1979), rabbit pulmonary macrophages (Kaplan & Nielson, 1979b), mouse peritoneal macrophages (Imber & Pizzo, 1981), rat hepatocytes and adipocytes (Glieman et al., 1983), and 3T3-L1 fibroblasts and adipocytes (Ney et al., 1984), bind and endocytose fast-form human α_2M. Fast-form α_2M produced by reaction with trypsin is bound and endocytosed identically with fast form produced by reaction with methylamine (Imber & Pizzo, 1981; Kaplan et al., 1981) or proteases from other categories (Feldman et al., 1983).

Fast-form α_2M that results from reaction of α_2M with plasmin is removed from the mouse circulation slightly more slowly than other fast forms of α_2M. In contrast to the first-order pattern of clearance of α_2M complexes with methylamine or trypsin, clearance of α_2M–plasmin is biphasic (Gonias et al., 1982). Although plasmin preferentially binds to α_2-antiplasmin under conditions of gross plasmin excess, such as may occur at sites of inflammation, α_2-antiplasmin is depleted, and α_2M becomes the major inhibitor of plasmin (Aoki et al., 1982). Since α_2M-bound plasmin retains 1–2% of its fibrinolytic activity (Harpel & Mosesson, 1973), binding, endocytosis, and degradation of the α_2M–plasmin complex are necessary for the complete inhibition of plasmin.

Experimental Procedures

Materials. The substrate H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide hydrochloride (S2251) was purchased from the Kabi Group. Porcine pancreatic elastase and trypsin were purchased from Worthington Biochemical Corp. Bovine pancreatic trypsin inhibitor (Trasylol) was purchased from FBA Pharmaceuticals and urokinase from Calbiochem Behring Corp. Sepharose 6B, phenylmethanesulfonyl fluoride (PMSFe, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethane sulfonic acid–hydrochloric acid (HEPES-HCl), bovine serum albumin (BSA), trypan blue, ϵ-aminocaproic acid (ϵACA), soybean trypsin inhibitor (SBTI), and methylamine (CH_3NH_2) were purchased from Sigma Chemical Co. Ultrogel ACA 22 was from LKB Instruments Inc. Outdated fresh frozen plasma was obtained from the Durham Veterans Administration Hospital Blood Bank. Earle's balanced salt solution (EBSS) (10× concentration) without phenol red and bicarbonate and either with or without Ca^{2+} and Mg^{2+} was obtained from GIBCO Laboratories. Also from GIBCO Laboratories were Hanks' balanced salt solution (HBSS), Neuman–Tytell serumless medium, Dulbecco's modified Eagle's medium, and fetal bovine serum. Brewers' thioglycolate broth was from Difco and Sepharose-coupled lactoperoxidase from P-L Biochemicals. $Na^{125}I$, carrier free, in 0.1 N NaOH was obtained from New England Nuclear and diluted 1:10 with 0.15 M sodium phosphate, pH 7.0, prior to use. All other reagents were the highest grade commercially available.

Preparation of Ligands. Human α_2M was prepared by affinity chromatography on Zn^{2+}–Sepharose (Kurecki et al., 1979) as modified by Imber & Pizzo (1981). This modification includes dialysis of fresh frozen plasma against deionized water for 72 h at 4 °C in order to precipitate fast-form α_2M (Imber & Pizzo, 1981; see Figure 2). Dialysis was followed by affinity chromatography on Zn^{2+}–Sepharose (Porath et al., 1975) and gel filtration chromatography on Ultrogel ACA 22.

Human plasminogen was prepared by affinity chromatography on lysine–Sepharose (Deutsch & Mertz, 1970) and activated to plasmin (PM) by incubation at 37 °C with 100 Plough units (P.U.) of urokinase per milligram of plasminogen (Pizzo et al., 1972).

Val_{442}-plasminogen was prepared by incubating 1 mg/mL plasminogen with 6 μg/mL porcine pancreatic elastase in the presence of 200 I.U./mL Trasylol in 0.02 M sodium phosphate and 0.15 M NaCl, pH 7.4 (PBS). After a 5-h digestion, the reaction was terminated with 1 mM PMSF, and the solution was chromatographed on lysine–Sepharose. Elastase elutes first as a small peak. The elution time of Val_{442}-plasminogen is slightly retarded, and it elutes in the second peak. The Kringle structures bind to the column and are eluted with 7.5 mM ϵACA (Castellino & Powell, 1981). Val_{442}-plasminogen was activated by incubation with 200 P.U. of urokinase per milligram of Val_{442}-plasminogen (Ney & Pizzo, 1982). The Val_{422}-plasmin so formed was less than 50% active.

α_2M–methylamine (α_2M–CH_3NH_2) complexes were prepared by incubating α_2M with 0.4 M methylamine in 50 mM tris(hydroxymethyl)aminomethane (Tris) and 150 mM sodium chloride, pH 8.0, overnight at room temperature. Unreacted methylamine was removed by dialysis against PBS (Imber & Pizzo, 1981).

α_2M–trypsin (α_2M–Trp) complexes were prepared by reaction of α_2M with excess trypsin at room temperature for 5 min. The reaction was terminated by addition of SBTI and 10 mM TLCK. Unreacted trypsin was removed by gel filtration chromatography on Sephadex G-150 (Imber & Pizzo, 1981).

α_2M–plasmin (α_2M–PM) complexes were prepared by incubation of excess plasmin with α_2M at 37 °C. The extent of the reaction was determined by SBTI-resistant cleavage of the plasmin substrate S2251 and was complete within 90 min. Unreacted plasmin was separated from the complex by gel filtration on Ultrogel ACA 22. Some plasmin complexes were further incubated with trypsin followed by SBTI and TLCK prior to gel filtration.

α_2M complexes with Val_{442}-plasmin (α_2M–Val_{442}-PM) were prepared as described for α_2M–trypsin complexes (Figure 1). Like reaction of trypsin with α_2M and unlike reaction of plasmin with α_2M, reaction of Val_{442}-plasmin with α_2M was complete in less than 5 min.

Figure 2 shows the results of native polyacrylamide gel electrophoresis of the various complexes. In each case, α_2M was completely converted from the "slow" to the "fast" form (Nelles et al., 1980). Unless otherwise specified, all subsequent experiments were performed with maximally reacted α_2M–protease.

Labeling of Complexes. Complexes were labeled with ^{125}I by the solid-state lactoperoxidase method (David & Reisfeld, 1974) as previously described (Imber & Pizzo, 1981).

Macrophage Primary Culture. Mouse peritoneal inflammatory macrophages were obtained by intraperitoneal injection of 1.0 mL of Brewers' thiolglycolate broth into black C57-B6 mice (Trudeau Institute). Macrophages were harvested 3 days later by peritoneal lavage, centrifuged at 250*g* for 10 min at 4 °C, resuspended in Dulbecco's modified Eagle's medium, and plated on Limbro 24-well plastic dishes at a density of 2.5×10^5 macrophages/cm^2. After 2–3 h at 37 °C, macrophages adhered to the plastic. Contaminating cells which remain in suspension were removed by washing the monolayer 3 times with 1.0 mL of HBSS. The cells were then covered with 1.0 mL of Neuman–Tytell serumless medium (Adams, 1979).

3T3-L1 Cell Culture. 3T3-L1 cells were obtained from the American Type Culture Collection. Cells were grown to confluence in Nunc 24-well multidishes using Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM glutamine (Coleman & Bell, 1980).

Macrophage Binding Studies. Macrophages in Neuman–Tytell serumless medium were placed at 4 °C for 0.5–1 h. The cells were washed 3 times with EBSS with Ca^{2+} and Mg^{2+}, 25 mM HEPES, and 20 mg/mL BSA, pH 7.3 (binding buffer A), or with EBSS without Ca^{2+} and Mg^{2+} 25 mM HEPES, 10 mg/mL BSA, and 5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.3 (binding buffer B). Cells were covered with 1.0 mL of binding buffer A or B and allowed to equilibrate for 2–3 h at 4 °C. At the beginning of the experiment, the buffer was aspirated, and radiolabeled complex in binding buffer A or B was placed on the cells. The reaction was terminated by aspiration of the ligand solution followed by three washes with 1.0 mL of binding buffer A or B. Cells were then washed 3 times with 1.0 mL of EBSS and 25 mM HEPES, pH 7.3 (Imber & Pizzo, 1981). Cell pellets were solubilized in 0.4 mL of 1% sodium dodecyl sulfate (SDS), and 0.1 N NaOH and counted in a Scientific Products AW 14120 γ counter. The protein content of the pellet was determined by the Peterson modification of the Lowry assay (Lowry et al., 1951; Peterson, 1977). Nonspecific binding was determined by incubating cells with radiolabeled ligand in the absence of Ca^{2+} and the presence of EDTA and was subtracted from total binding to give specific binding.

3T3-L1 Fibroblast Binding Studies. The binding assay for the 3T3 cell monolayers was a modification of the macrophage binding assay (Imber & Pizzo, 1981; Ney et al., 1984). Cells were placed at 4 °C for 15–30 min and then washed 4 times with 1.0 mL of binding buffer A. After equilibration at 4 °C for 2–4 h, the binding buffer was aspirated, and a solution of radiolabeled ligand in binding buffer was placed on the cells. After incubation with ligand, the binding reaction was terminated by aspiration of the ligand solution and three washes with 1.0 mL of binding buffer A followed by three washes with 1.0 mL of EBSS with 25 mM HEPES, pH 7.3. Cells were harvested and counted as described for macrophages. Nonspecific binding, determined by incubation of ligand with 5 mM EDTA in the binding buffer, was the same as that determined by inclusion of a 100-fold excess of unlabeled ligand (Ney et al., 1984). However, since incubation of cells with EDTA caused loss of 3T3 cells from the monolayer, nonspecific binding was determined by incubation of radiolabeled ligand in the presence of a 100-fold molar excess of unlabeled ligand. In all cases, nonspecific binding was less than 20% of the total binding.

Uptake Studies. Macrophage monolayers were washed 3 times with binding buffer A or B at 37 °C and equilibrated at 37 °C for 1–2 h. Reaction was begun by addition of radiolabeled ligand in a solution of binding buffer A or B and terminated by aspiration of the ligand solution and three washes with 1.0 mL of binding buffer A or B at 4 °C. The pellets were washed 3 times with EBSS and 25 mM HEPES, pH 7.3 at 4 °C, and the pellets were solubilized, γ counted, and measured for protein content as described above. Nonspecific uptake was measured in the presence of 5 mM EDTA and subtracted from total uptake to determine specific uptake.

Clearance Studies. A solution of ^{125}I-labeled ligand in 0.2–0.4 mL of PBS was injected into the lateral tail vein of a CD-1 female mouse. Twenty-five-microliter blood samples, obtained by insertion of a heparinized capillary tube into the retro-orbital venous plexus, were counted for γ radioactivity. The radioactivity in the blood sample obtained 5 s after injection of the ligand was defined as the initial ligand concentration, and the radioactivity in succeeding samples was expressed as a percent of this first measurement (Imber & Pizzo, 1981).

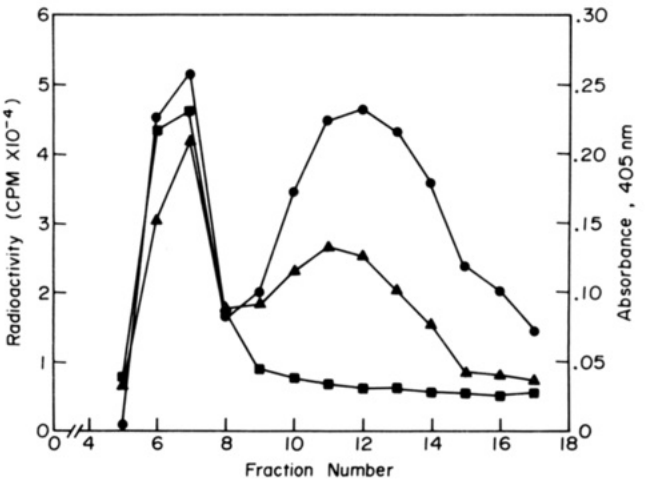

FIGURE 1: Gel filtration elution profile of products of reaction of Val_{442}-plasmin with α_2M. α_2M was incubated with excess ^{125}I-Val_{442}-plasmin for 5 min at 37 °C in a total volume of 0.5 mL and the reaction mixture subjected to gel filtration on a 27-mL Sephadex G-150 column (0.7 cm × 20 cm). Fractions (0.5 mL) were collected and assayed for γ radioactivity (●) and S2251 amidolytic activity in the presence (■) and absence (▲) of excess SBTI.

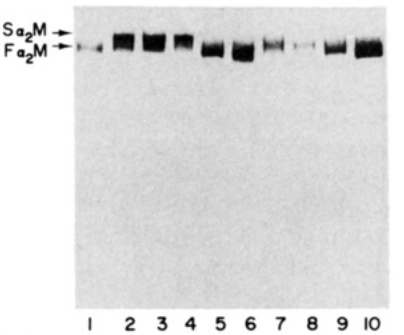

FIGURE 2: Native polyacrylamide gel electrophoresis of α_2M complexes. Complexes of α_2M with different proteases or CH_3NH_2 were prepared as described under Experimental Procedures and were examined on native polyacrylamide (5%) gels. Lane 1, ^{125}I-α_2M–CH_3NH_2; lanes 2 and 3, α_2M after gel filtration on Ultrogel ACA 22 without dialysis against deionized H_2O; lane 4, α_2M as in lanes 2 and 3 with dialysis against deionized H_2O; lane 5, α_2M–CH_3NH_2 (40 μg); lane 6, α_2M–Trp; lane 7, α_2M–PM; lane 8, trypsin-treated α_2M–PM; lane 9, α_2M–Val_{442}-PM; lane 10, α_2M–CH_3NH_2 (100 μg).

RESULTS

Preparation of α_2M–Val_{422}-PM. α_2M was incubated with an excess of ^{125}I-Val_{422}-PM, and the resulting complexes were subjected to gel filtration chromatography on Sephadex G-150. A typical column profile is shown in Figure 1. α_2M–^{125}I-Val_{422}-PM elutes first and is well separated from free ^{125}I-Val_{422}-PM which elutes in the second peak. The complex retains its amidolytic activity toward S2251 even in the presence of SBTI, but the amidolytic activity of the free protease is completely inhibited by SBTI. The ratio of amidolytic activity to radioactivity of α_2M-bound protease is higher than that of free protease as would be expected since only active protease binds to α_2M (Barrett & Starky, 1973). Native gel electrophoresis of the α_2M–Val_{442}-plasmin complex (Figure 2) shows the slow to fast conformational change accompanying reaction of α_2M with protease. These results are those that would be predicted from results of experiments with numerous other proteases (Barrett & Starkey, 1973; Starkey & Barrett, 1977).

Figure 3 shows the results of the experiment in which increasing amounts of ^{125}I-Val_{422}-PM were added to a constant amount of α_2M and the resulting complexes subjected to gel

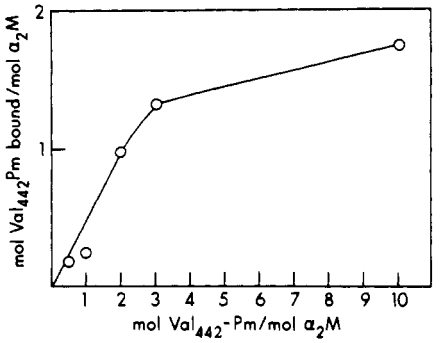

FIGURE 3: Stoichiometry of Val_{442}-plasmin binding to α_2M. Increasing amounts of ^{125}I-Val_{442}-plasmin were incubated with constant amounts of α_2M. Bound protease was separated from free protease by gel filtration chromatography on Sephadex G-150. The amount of Val_{442}-plasmin bound to α_2M was determined by γ counting and Lowry protein assays of the complexes.

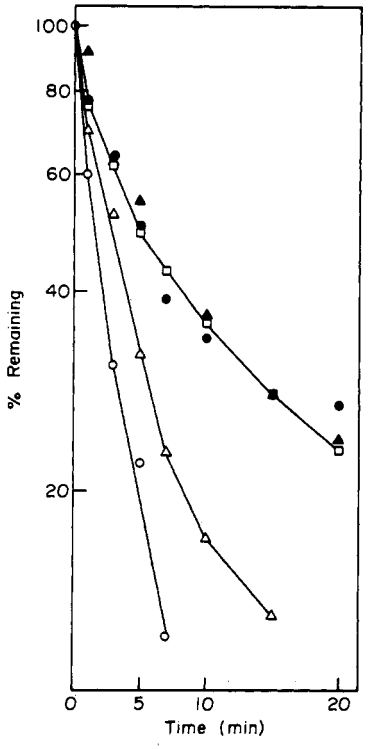

FIGURE 4: Clearance of α_2M–Val_{442}-PM complexes with varying degrees of saturation with protease. α_2M was incubated with increasing amounts of ^{125}I-Val_{442}-PM, and α_2M-bound ^{125}I-Val_{442}-PM was separated from free ^{125}I-Val_{442}-PM. The resulting complexes were injected into mice. The radioactivity remaining in the mouse circulation is plotted as a function of time for α_2M–^{125}I-Val_{442}-PM complexes with ratios (moles of Val_{442}-PM to moles of α_2M) of 0.18 (▲), 0.24 (●), 0.98 (□), 1.33 (△), and 1.74 (○).

filtration chromatography. The amount of ^{125}I-Val_{422}-PM bound to α_2M, determined from the γ radioactivity of the complexes and the specific activity of the labeled protease, is plotted as a function of the amount of ^{125}I-Val_{422}-PM in the incubation. The initial slope of this line is 0.5 mol of Val_{442}-PM bound per mole in the incubation. These results are consistent with activity of Val_{442}-PM of 50%. The maximum amount of ^{125}I-Val_{422}-PM bound to α_2M is 2 mol/mol of α_2M. This maximum binding occurs at 8–10 mol of ^{125}I Val_{442}-PM/mol of α_2M, i.e., 4–5 mol of active ^{125}I-Val_{442}-PM/mol of α_2M. These results are very similar to those obtained by Sottrup-Jensen et al. (1981c) in their experiments with trypsin and α_2M.

Clearance of α_2M–^{125}I-Val_{422}-PM (Prepared at Different Molar Ratios of Protease to Inhibitor). The complexes

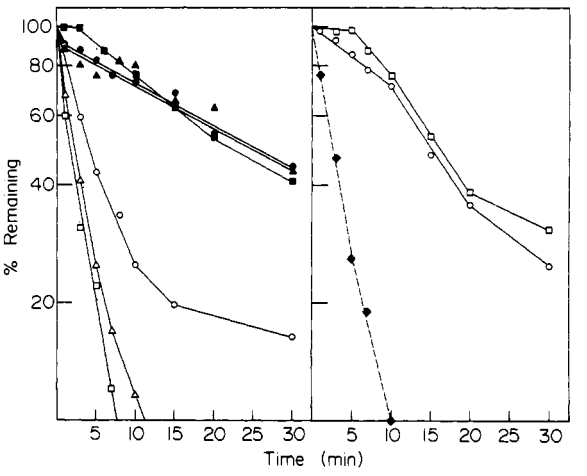

FIGURE 5: Clearance of α_2M–protease complexes. (Left panel) The disappearance of labeled complexes was measured as a function of time for α_2M–Val_{442}-PM (△), α_2M–PM (○), and trypsin-treated α_2M–PM (□). The closed symbols show the clearance of these same complexes in the presence of a 1000-fold molar excess of unlabeled α_2M–CH_3NH_2. (Right panel) Disappearance of ^{125}I-α_2M–CH_3NH_2 from the mouse circulation as a function of time (♦). Solid lines represent the clearance of ^{125}I-α_2M–CH_3NH_2 in the presence of a 1000-fold molar excess of α_2M–Val_{442}-PM (□) and α_2M–PM (○).

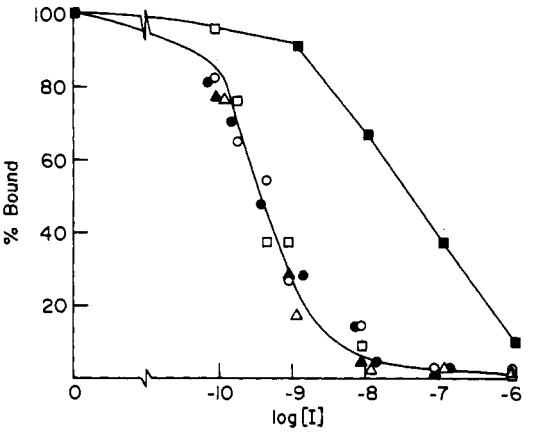

FIGURE 6: Inhibition of binding of α_2M–CH_3NH_2 to macrophages by various α_2M–protease complexes. Macrophage monolayers were incubated with 0.1 nM ^{125}I-α_2M–CH_3NH_2 at 4 °C in the presence of varying concentrations of native α_2M (■), α_2M–CH_3NH_2 (▲), α_2M–Trp (△), α_2M–PM (●), trypsin-treated α_2M–PM (○), and α_2M–Val_{442}-PM (□). Binding is plotted as the percent of binding without inhibitors as a function of log inhibitor concentration. Each point is the mean of triplicate determinations.

formed by reaction of α_2M with varying amounts of ^{125}I-Val_{422}-plasmin were injected into mice. Since the complexes were subjected to gel filtration chromatography, only α_2M-bound ^{125}I-Val^{442}-PM was injected. The disappearance of these complexes from the mouse circulation is shown as a function of time in Figure 4. Since only the Val_{442}-PM was labeled, these experiments showed the clearance of α_2M with 1 or 2 mol of ^{125}I-Val_{442}-PM only. Fully saturated α_2M, α_2M–(^{125}I-Val_{422}-PM)$_2$, disappears from the circulation of a first-order reaction iwth a $t_{1/2}$ of 3 min. In contrast, less saturated complexes, mixtures of α_2M–^{125}I-Val_{442}-PM and α_2M–(^{125}I-Val_{422}-PM)$_2$, clear in a slower biphasic reaction.

Clearance of α_2M Fast Forms. Figure 5 shows the mouse plasma clearance of various α_2M complexes. ^{125}I-α_2M–PM clears with a slightly longer half-life and has a second slower component to its clearance, confirming previous results from this laboratory (Gonias et al., 1982). In contrast, ^{125}I-α_2M–CH_3NH_2, ^{125}I-α_2M–Val_{442}-PM, and ^{125}I-labeled trypsin-treated α_2M–PM all cleared identically in a first-order reaction

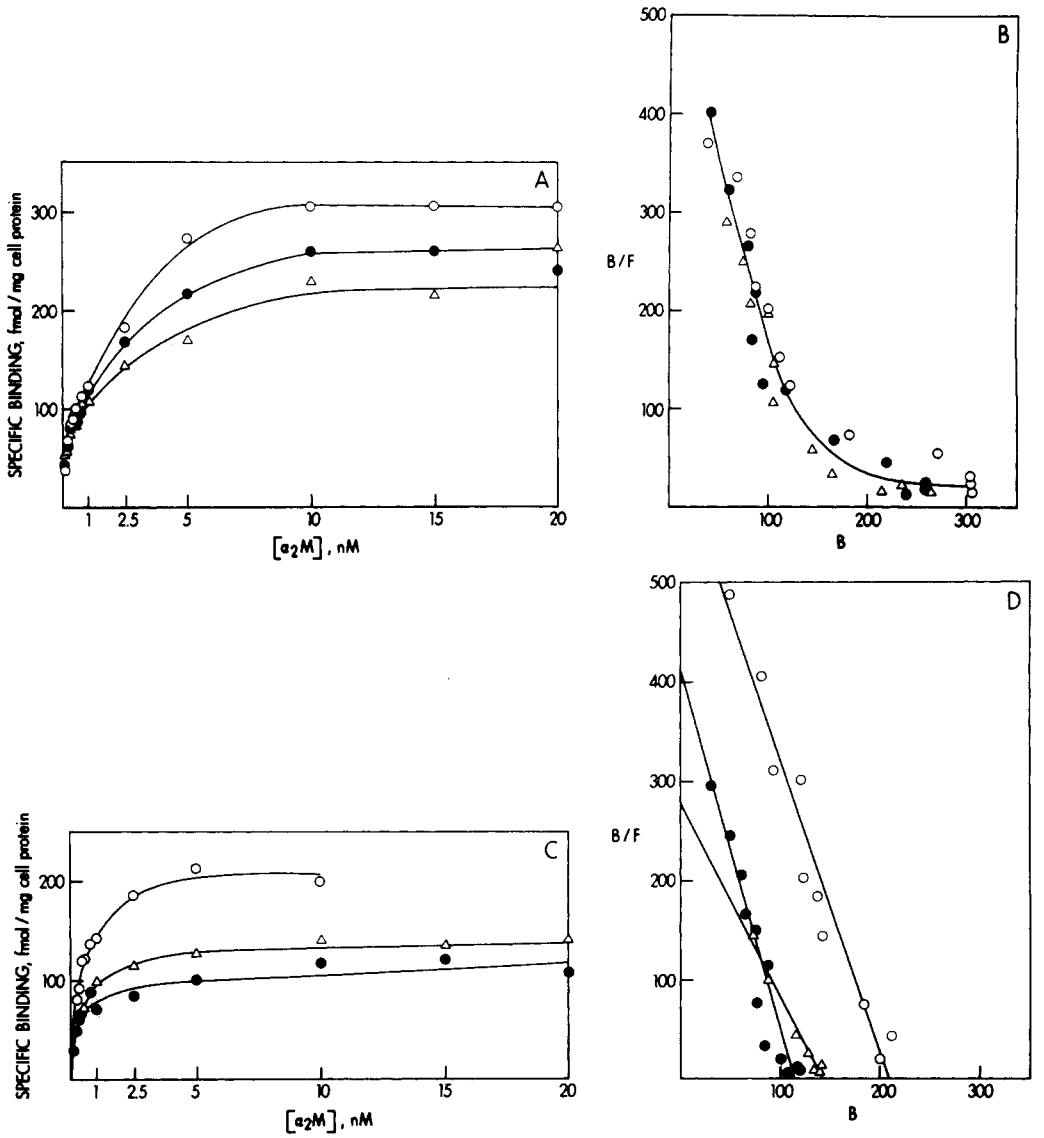

FIGURE 7: Comparison of the binding of α_2M–CH_3NH_2, α_2M–PM, and trypsin-treated α_2M–PM to macrophages and fibroblasts. Various concentrations of ^{125}I-α_2M–CH_3NH_2 (△), ^{125}I-α_2M–PM (●), or ^{125}I-labeled trypsin-treated α_2M–PM (○) were incubated with mouse peritoneal macrophages (panels A and B) or 3T3-L1 fibroblasts (panels C and D) at 4 °C. Nonspecific binding was determined by incubation of labeled ligand in the presence of EDTA for macrophages or a 100-fold molar excess of unlabeled α_2M–CH_3NH_2 for fibroblasts and subtracted from total binding to determine specific binding. Specific binding is shown as a function of concentration in panels A and C and is plotted according to the method of Scatchard (1949) in panels B and D. Points shown are the mean of triplicate determinations.

with $t_{1/2}$ = 3 min. Plasma clearance of all the complexes was inhibited by a 1000-fold molar excess of unlabeled α_2M–CH_3NH_2. Similarly, the clearance of ^{125}I-α_2M–CH_3NH_2 was inhibited by a 1000-fold molar excess of unlabeled α_2M–PM or α_2M–Val_{442}-PM.

Inhibition Studies. We examined the in vitro binding of the complexes to determine whether this slower clearance was due to altered receptor recognition of α_2M–PM. Figure 6 shows the results of incubating 0.1 nM ^{125}I-α_2M–CH_3NH_2 with macrophage monolayers at 4 °C in the presence of varying concentrations of unlabeled complexes, α_2M–CH_3NH_2, α_2M–Trp, α_2M–PM, trypsin-treated α_2M–PM, and α_2M–Val_{422}-PM. These complexes all inhibit the binding of ^{125}I-α_2M–CH_3NH_2 to the same extent at the same concentrations; 50% inhibition occurs at about 0.4 nM concentration of each complex. In contrast, native α_2M inhibits the binding of 0.1 nM ^{125}I-α_2M–CH_3NH_2 with 100-fold less affinity than α_2M–protease complexes.

On Times and Off Times. ^{125}I-Labeled complexes of α_2M with CH_3NH_2, plasmin, or Val_{442}-plasmin were incubated with macrophage monolayers at 4 °C for varying times. For all three complexes, a steady state of binding occurred between 8 and 10 h. ^{125}I-α_2M–CH_3NH_2 was incubated at 4 °C with macrophage monolayers for 12 h. The labeled ligand was removed, and the macrophages were incubated with binding buffer alone or with binding buffer and excess unlabeled ligand for varying times. Even after 24 h, only 4% of the labeled ligand had dissociated from the macrophages in the incubation with binding buffer alone and only 16% in the incubation with binding buffer and excess unlabeled ligand.

Binding Isotherms. We next compared the 4 °C binding of labeled complexes as a function of concentration. These results are shown in Figure 7. The specific binding of ^{125}I-α_2M–CH_3NH_2, ^{125}I-α_2M–PM, and trypsin-treated ^{125}I-α_2M–PM to both mouse peritoneal macrophages and 3T3-L1 fibroblasts was the same. A Scatchard plot (Scatchard, 1949) of the specific binding data for macrophages did not yield a straight line; however, that for 3T3-L1 fibroblasts did yield a straight line with an apparent K_d of 0.48 nM and a receptor activity of 140 fmol/mg of cell protein for α_2M–CH_3NH_2, an

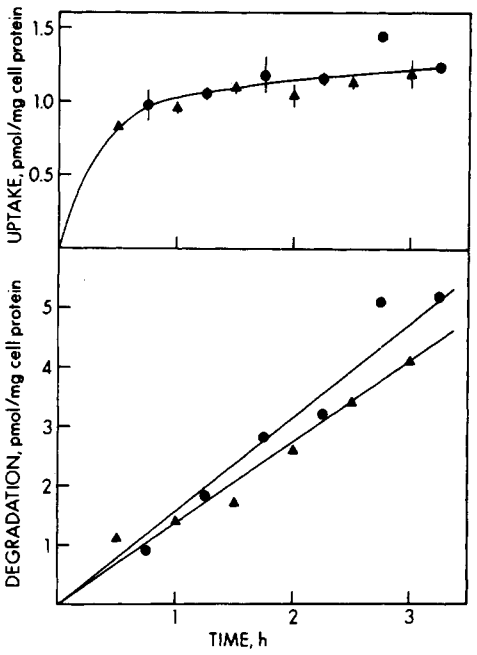

FIGURE 8: Time course of uptake and degradation of α_2M–CH_3NH_2 and α_2M–PM by macrophages. Fifty nanomolar ^{125}I-α_2M–CH_3NH_2 (▲) or ^{125}I-α_2M–PM (●) was incubated with macrophage monolayers at 37 °C for varying times. Reaction was terminated by removal of the ligand solution and precipitation in 10% (final volume) Cl_3CCOOH. Cl_3CCOOH-soluble material is plotted as a function of time in the bottom panel. Cell pellets were washed, harvested, and counted for γ radioactivity. Pellet-associated radioactivity is plotted as a function of time in the top panel. Points shown are the means of triplicate determinations.

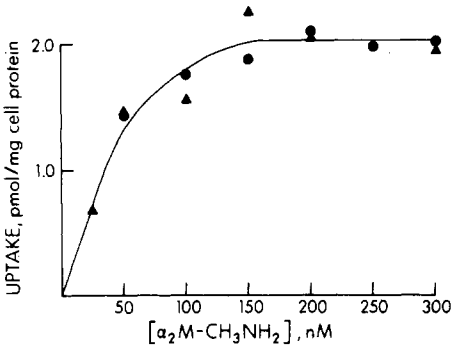

FIGURE 9: Concentration dependence of uptake of α_2M–CH_3NH_2 and α_2M–PM complexes by macrophages. Macrophage monolayers were incubated with various concentrations of ^{125}I-α_2M–CH_3NH_2 (▲) or ^{125}I-α_2M–PM (●) for 3 h at 37 °C. Cell pellet associated radioactivity is plotted as a function of concentration. Points shown are the mean of triplicate determinations.

apparent K_d of 0.29 nM and a receptor activity of 110 fmol/mg of cell protein for α_2M–PM, and an apparent K_d of 0.35 nM and a receptor activity of 210 fmol/mg of cell protein for trypsin-treated α_2M–PM.

Uptake Studies. Figures 8 and 9 compare the uptake of α_2M–CH_3NH_2 and α_2M–PM complexes at 37 °C. Fifty nanomolar ^{125}I-labeled ligand was incubated with macrophages for varying periods of time. Uptake, as measured by cell-associated radioactivity, and degradation, as measured by trichloroacetic acid (Cl_3CCOOH) soluble radioactivity in the medium, were the same for both α_2M–CH_3NH_2 and α_2M–PM (Figure 8). Steady state was achieved for both ligands after 1–2 h. The cell-associated ligand was measured as a function of concentration and was the same for both ligands (Figure 9). A double-reciprocal plot of these data shows that maximum uptake is 2.0 pmol/mg of cell protein and that the concentration of ligand giving half-maximal uptake is 50 nM.

Discussion

Previous studies from this laboratory have shown a sutble but reproducible difference between the clearance of fast-form α_2M resulting from reaction with plasmin and fast-form α_2M resulting from reaction with trypsin or methylamine (Gonias et al., 1982). Such a difference would be important at sites of inflammation where α_2M becomes the major inhibitor of plasmin (Aoki et al., 1978) because binding, endocytosis, and degradation of the α_2M–plasmin complex are necessary for the complete inhibition of plasmin by α_2M (Harpel & Mosesson, 1973).

Fragments of plasmin, such as Val_{422}-plasmin, which retain the active site of plasmin but lack the "Kringle" structures (Sottrup-Jensen et al., 1978), may be generated by the leukocyte elastase and plasminogen activator present at sites of inflammation (Moroz, 1981; Werb, 1982; Ney & Pizzo, 1982). Moroz (1981) has shown that the "mini-plasmin" produced by leukocyte elastase cleavage of plasminogen is more susceptible to inhibition of α_2M and less susceptible to inhibition by α_2-anti-plasmin than plasmin. Our results confirm this oservation with Val_{442}-plasmin and α_2M. Val_{442}-plasmin reacts with α_2M more rapidly than plasmin. In addition, Val_{442}-plasmin binds to α_2M with a stoichiometry of 2 mol of protease to 1 mol of α_2M and plasmin with a stoichiometry of 1 to 1 (Iwamoto & Abiko, 1970; Sugihara et al., 1971; Pochon et al., 1978). Unlike α_2M–PM, α_2M–Val_{442}-PM is removed in a first-order reaction, $t_{1/2}$ = 3 min, from the mouse circulation.

We initially postulated that this difference between the removal of α_2M–PM from mouse circulation and the clearance of other fast-form α_2M species might be due to incomplete exposure of the receptor recognition site on α_2M since plasmin binds to α_2M with a stoihciometry of 1 to 1 (Pochon et al., 1978) and cleaves only two of the four subunits of α_2M (Howell et al., 1983). This hypothesis was supported by the rapid clearance of α_2M–Val_{442}-PM, which has a stoichiometry of 2 to 1, and trypsin-treated α_2M–PM, in which all four of the α_2M subunits are cleaved although trypsin does not bind to the complex (Howell et al., 1983), and by the slower clearance of unsaturated α_2M–^{125}I-Val_{442}-PM complexes. However, studies in vitro with mouse peritoneal macrophages and 3T3-L1 fibroblasts showed no difference in the specific binding of α_2M–CH_3NH_2, α_2M–PM, trypsin-treated α_2M–PM, and α_2M–Val_{442}-PM. Uptake studies in mouse peritoneal macrophages again showed no difference in the specific uptake of α_2M–CH_3NH_2 and α_2M–PM. These data are not consistent with the hypothesis that the slower clearance of α_2M–PM is due to altered receptor recognition of the complex. It is possible that this altered clearance is due to interaction between the α_2M–PM complex and some factor in mouse blood. Plasmin in the α_2M–PM complex can cleave fibrin (Harpel & Mosesson, 1973) and bind anti-plasmin antibodies (Cummings & Castellino, 1984) and so could interact with fibriongen or some other blood component and reduce the availability of α_2M–PM for binding and endocytosis.

The complexes all inhibit the binding of 0.1 nM ^{125}I-α_2M–CH_3NH_2 to the same extent at the same concentration. Since the concentration of ^{125}I-α_2M–CH_3NH_2 is well below its K_d, the concentration of inhibitor needed to decrease binding by 50% is an approximation of the K_d of the inhibitor. For all the complexes, this K_d is 0.4 nM. Native α_2M inhibits binding slightly, probably because of contamination of native α_2M preparations with some fast form (Van Leuven et al., 1979).

The Scatchard plot of the 4 °C isotherm for mouse peritoneal macrophages was curved. Previous studies from this

laboratory examining only the higher (Imber & Pizzo, 1981) or lower (Feldman et al., 1983) concentrations showed linear Scatchard plots. The Scatchard plot of the 4 °C isotherm for 3T3-L1 fibroblasts was linear even when concentrations up to 50 nM were included in the study (Ney et al., 1984). Nonlinear Scatchard plots of α_2M isotherms have been reported by other groups (Dickson et al., 1981).

The inability to demonstrate dissociation of bound ligand has also been reported by Kaplan, who suggests that this receptor system does not consist of independent equivalent binding sites. Rather, receptors and ligands are probably multivalent so that one receptor may interact with more than one molecule of ligand or, conversely, one molecule of ligand may react with several different receptors. Thus, interpretation of a nonlinear Scatchard plot in this nonideal system is impossible; nonlinearity may reflect receptor orientation or density rather than the existence of two different classes of receptor (Kaplan, 1981).

These studies show that the slower clearance of α_2M–PM is not due to impaired receptor recognition of the complex but may be due to α_2M–PM interaction with some factor in mouse blood. This interaction is eliminated by reaction of α_2M–PM with trypsin and does not take place for α_2M–Val_{442}–PM. The endocytosis and degradation of α_2M–PM and α_2M–Val_{442}–PM by macrophages and fibroblasts probably occur in vivo at sites of inflammation.

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