Identification of a Monoclonal Antibody Specific for a Neoantigenic Determinant on α_2-Macroglobulin: Use for the Purification and Characterization of Binary Proteinase–Inhibitor Complexes†

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ABSTRACT: A monoclonal antibody was obtained from the fusion of spleen cells of mice, immunized with methylamine-treated α_2-macroglobulin (α_2M), with the myeloma cell line P3-X63-Ag8.653. A competitive binding assay demonstrated that the antibody was specific for a neoantigen expressed on α_2M when the inhibitor reacts with proteinases or with methylamine. When immobilized, the monoclonal antibody retained its ability to specifically bind α_2M–proteinase complexes or methylamine-treated α_2M, both of which could be quantitatively recovered from the immunoaffinity column by lowering the pH to 5.0. Binary α_2M–proteinase complexes of trypsin, plasmin, and thrombin, prepared by incubating large amounts of α_2M with a small amount of enzyme, were isolated by immunoaffinity chromatography. Each purified complex was characterized with regard to proteinase content, extent of α_2M subunit cleavage, extent of thiol ester hydrolysis, and extent of conformational change. Each complex contained 0.8–0.9 mol of proteinase/mol of inhibitor. In the α_2M–thrombin, α_2M–plasmin, and α_2M–trypsin complexes, approximately 50%, 60%, and 75% of the subunits are cleaved, respectively. Titration of sulfhydryl groups revealed that all purified binary complexes contained 2 ± 0.5 mol of thiol/mol of complex, suggesting that each complex retains two intact thiol ester bonds. When the purified complexes were incubated with excess trypsin or with methylamine, an additional 1–2 mol of sulfhydryl/mol of complex could be titrated. The extent of the conformational change in these isolated complexes was examined by measuring enhancement of the fluorescence of 6-(*p*-toluidino)-2-naphthalenesulfonic acid and by measuring electrophoretic mobility under native conditions. In all complexes, the fluorescence enhancement was between that observed for native α_2M and that observed for the ternary α_2M–trypsin complex. Likewise, the electrophoretic mobility of the binary complexes was intermediate between that of native α_2M and that of the ternary α_2M–trypsin complex. Thus, formation of a 1:1 α_2M–proteinase complex results in alterations largely confined to one of two functional units within the inhibitor. However, the 1:1 complex only binds between 0.3 and 0.4 mol of additional proteinase. These results suggest that some rearrangement of the binary complex occurs, reducing the ability of the complex to bind additional proteinase molecules. A conformational alteration of the complex once 1 mol of proteinase is bound might be a key factor in determining the binding stoichiometry of α_2M for various proteinases.

α_2-Macroglobulin (α_2M)[1] is a large molecular weight (M_r 718 000) plasma glycoprotein that contains four identical subunits covalently associated in pairs via disulfide bonds (Swenson & Howard, 1979; Hall & Roberts, 1978; Sottrup-Jensen et al., 1984). The molecule functions as a proteinase inhibitor and is inactivated by small amines, such as methylamine. This reaction results in the covalent incorporation of the amine into the inhibitor (Swenson & Howard, 1979) with the liberation of free sulfhydryl groups (Salvesen et al., 1981), and a conformational change in the molecule (Gonias et al., 1982; Björk & Fish, 1982; Barrett et al., 1979). On the basis of these and other studies, it has been proposed that α_2M contains internal thiol ester bonds formed from side chains of cysteinyl and glutamyl residues on the polypeptide chain (Sottrup-Jensen et al., 1984; Howard, 1981), a feature shared with two complement proteins, C3 and C4 (Thomas et al., 1982; Cambell, 1981).

As a proteinase inhibitor, α_2M exhibits a broad specificity. While the interaction of a number of different enzymes with this inhibitor has been examined, the detailed sequence of events which lead to inhibition of the proteinase is largely unknown at this time. A description of the reaction mechanism has been hampered by the lack of a clear understanding of the nature of the product formed when a proteinase reacts with α_2M. For example, the extent of polypeptide chain cleavage, sulfhydryl appearance, and conformational change that occurs when a single proteinase molecule associates with α_2M has not been definitively established. Studies by Christensen and Sottrup-Jensen (1984) suggest that the stoichiometry of these reactions depends upon the initial concentrations of proteinase and inhibitor. Their data suggest that at lower concentrations of trypsin the association of 1 mol of proteinase with α_2M results in the cleavage of a single subunit and the appearance of two free sulfhydryl groups. At higher concentrations of trypsin (between 0.02 and 0.5 μM with α_2M levels in the

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[1] Abbreviations: α_2M, α_2-macroglobulin; 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; SBTI, soybean trypsin inhibitor; DFP, diisopropyl fluorophosphate; HPLC, high-pressure liquid chromatography; S-2238, H-D-Phe-Pip-Arg-*p*-nitroanilide; S-2251, D-Val-Leu-Lys-*p*-nitroanilide; S-2222, Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane; DMF, dimethylformamide; kDa, kilodalton(s).

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micromolar range), their data indicate that the association of a single mole of proteinase results in the cleavage of two polypeptide chains and four thiol ester bonds. On the other hand, titration studies by Björk et al. (1984) suggest that at both high and low concentrations of proteinase and inhibitor, the association of a single mole of proteinase with the inhibitor results in the cleavage of two α_2M subunits and the appearance of two sulfhydryl groups. These discrepancies most likely stem from the difficulty in assessing the properties of 1:1 complexes in mixtures containing native α_2M and 1:1 and 1:2 proteinase complexes.

The present studies were undertaken to determine the stoichiometry of individual reactions that occur when a single proteinase associates with α_2M and to characterize the extent of the conformational change occurring in binary α_2M–proteinase complexes. The results demonstrate that in all binary complexes examined, between two and three of the four α_2M subunits are cleaved, while two free sulfhydryl groups are available for titration with DTNB. In addition, the conformational change is incomplete and is about 50% of that observed for ternary α_2M–proteinase complexes containing two proteinase molecules. The results are consistent with the concept that α_2M contains two functional units and suggest that the association of 1 mol of proteinase with the inhibitor largely results in alterations in one functional unit. However, the 1:1 complex could only bind between 0.3 to 0.4 mol of proteinase, suggesting that a rearrangement of the complex occurs which reduces its ability to bind additional enzyme molecules.

Materials and Methods

Materials

Affi-Gel 10 and DEAE Affi-Gel Blue were purchased from Bio-Rad. H-D-Phe-Pip-Arg-*p*-nitroanilide (S-2238), D-Val-Leu-Lys-*p*-nitroanilide (S-2251), and Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (S-2222) was obtained from Helena. Human cryosupernatant was obtained from the Washington Regional Blood Services, American Red Cross. Diisopropyl fluorophosphate (DFP) and *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (NPGB) were purchased from Sigma, St. Louis, MO, while trypsin was obtained from Calbiochem.

Methods

Proteins. 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). 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. 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). 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.

Protein Concentrations. These were determined spectrophotometrically by using the following values for $E_{1\%}^{280nm}$ 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). Protein concentrations were corrected for light scattering as described (Prendergast & Mann, 1977). The concentrations stated for all proteinases and α_2M are based on their active-site concentrations.

Monoclonal Antibody Production. Male BALB/c mice were immunized with three consecutive injections of methylamine-treated α_2M. The immunization schedule involved an initial intraperitoneal injection of the purified antigen (50 μg) in complete Freund's adjuvant followed by an intraperitoneal injection of purified antigen (50 μg) in incomplete Freund's adjuvant after 2 weeks. Following the second injection, the mouse was bled and the titer determined. A third intraperitoneal injection, consisting of purified antigen (50 μg) in saline, was given at week 4. Three days after the final injection, spleen cells (1×10^8) from immunized mice were fused with the mouse myeloma cell line (1×10^7) P3-X63-Ag8.653 (Kearney et al., 1979) in 50% poly(ethylene glycol 4000) (Merck) by the method of Kohler and Milstein (1975). The fused cells were suspended in hypoxanthine- and thymidine-containing medium overnight, then resuspended in hypoxanthine–aminopterin- and thymidine-containing medium, and distributed into eight microtiter plates. Supernatants were assayed after 15–20 days. The selected positive cultures were cloned twice by the limiting dilution method.

Screening Assay. Cell supernatants were assayed by using a solid-phase enzyme-linked immunoabsorbent assay. Purified methylamine-treated α_2M was coated to the wells of microtiter plates (Immunolon 2, Dynatech) at a concentration of 1 μg/mL in 50 mM $NaHCO_3$, pH 9.5, overnight at 4 °C. After the plates were washed, they were coated with bovine serum albumin (10 mg/mL) in the same buffer for 1 h at 37 °C. The plates were washed and then incubated with cell supernatants in a 50 mM sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl and 0.02% Tween 20. Binding of antibody to the antigen-coated wells was detected by adding an anti-mouse IgG–alkaline phosphatase conjugate. Competitive binding assays were performed by preincubating the cell supernatants or purified antibodies with antigen for 60 min at 37 °C prior to addition to the wells. Large amounts of antibody were obtained by production of ascites fluid. After the ascites fluid was obtained, the fluid was subjected to centrifugation, and the supernatant was stored at −70 °C prior to purification of the IgG fraction.

Purification of the IgG Fraction from the Ascites Fluid. The IgG fraction from the ascites fluid was purified by one of two methods. The first method, used for the purification of large amounts of IgG, involved ammonium sulfate precipitation followed by dialysis versus 10 mM Tris, pH 7.6, and chromatography on a DEAE Affi-Gel Blue column equilibrated in the same buffer. A sharp peak at the void volume was often cloudy and contained little IgG. The antibody was slightly retarded by the column and eluted as a broad peak. SDS–PAGE indicated that the peak contained greater than 95% IgG. A second purification method involved chromatography on a protein A–Sepharose affinity column. The 1.0 × 6 cm column was equilibrated in 140 mM sodium phosphate, pH 8.2. The ascites fluid was diluted 5-fold in the equilibration buffer and applied to the column. After the eluent absorbance had returned to base line, the buffer was changed to 0.1 M sodium citrate, pH 6.0, to elute the IgG_1 fraction.

Affinity Chromatography of α_2M–Proteinase Complexes. The antibody purified by chromatography on the DEAE Affi-Gel Blue column was coupled to Affi-Gel 10 in a buffer of 0.1 M HEPES/80 mM $CaCl_2$, pH 7.5, for 6 h at room

temperature. A ratio of 2 mg of antibody/mL of gel was used. Following incubation, residual coupling sites were blocked by adding 0.1 mL of 1 M glycine ethyl ester/mL of gel and incubating for 1 h. The amount of antibody coupled to the gel was determined by measuring the absorbance of the reaction supernatant after adjustment of the pH to 4 with acetic acid. The affinity resin was placed into a 0.5 cm × 20 cm column and washed with 1 L of 20 mM Tris/0.5 M NaCl, pH 7.4, and then equilibrated with 50 mM sodium phosphate/0.5 M NaCl, pH 7.4, prior to use. All chromatography experiments were conducted at room temperature in a buffer of 50 mM sodium phosphate/0.5 M NaCl, pH 7.4. Chromatography was performed at a flow rate of 0.25 mL/min. After application of the sample, the column was washed with elution buffer, followed by a buffer of 50 mM sodium acetate, pH 5.0. The complexes were eluted from the column by using a buffer of 50 mM sodium acetate/0.5 M NaCl, pH 5.0.

Electrophoresis. SDS–PAGE was performed on a 8–25% gradient gel and was run and stained with Coomassie Blue on a Pharmacia PhastSystem electrophoresis apparatus. Tris–borate/PAGE was performed as described by Nelles et al. (1981).

Free Sulfhydryl Determinations. Titration of sulfhydryl groups was performed with 1–2 μM α_2M–proteinase complex using 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). Sulfhydryl group were also titrated with the fluorescent probe *N*-(1-pyrenyl)maleimide. For these measurements, excitation was at 346 nm, while emission was monitored at 387 nm. *N*-(1-Pyrenyl)maleimide was removed from a stock solution (1.5 mM in DMF) and added to a cuvette containing buffer such that the final concentration was 50 μM. The base-line fluorescence was recorded, and 40 nM α_2M–proteinase complex was added at time zero. The fluorescence change was monitored for approximately 10 min. A rapid burst was followed by a gradual linear increase, which was extrapolated to zero time to obtain the fluorescence change. The change in fluorescence signal was converted to moles of thiol present by construction of a standard curve using a freshly prepared ternary α_2M–trypsin complex as the standard. The thiol content of the standard was measured by titration with DTNB.

TNS Fluorescence Measurements. Measurements of TNS fluorescence were performed in a buffer of 50 mM HEPES/150 mM NaCl, pH 8.0, on an SLM-8000 fluorometer. The excitation wavlength was 315 nm, and the excitation slit was 2 nm while the emission was 16 nm. The spectra were recorded at room temperature, and the concentration of TNS was 50 μM.

Measurement of Bound Proteinase Activity. Bound proteinase activity was measured both in the presence and in the absence of a 5–10-fold molar excess of SBTI (over proteinase) using the chromogenic substrates Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (S-2222) for trypsin, H-D-Phe-Pip-Arg-*p*-nitroanilide (S-2238) for thrombin, and D-Val-Leu-Lys-*p*-nitroanilide (S-2251) for plasmin. When activity measurements were used to determine the recovery of α_2M–proteinase complex from the affinity column, a standard was prepared by reacting the appropriate enzyme with a 5-fold molar excess of α_2M. The activity of the purified binary complex was compared to that of the standard to calculate activity. Comparison of kinetic parameters for the hydrolysis of the substrates by the purified binary complexes with those of the standards ensured that no changes in these parameters occurred upon purification of the complex.

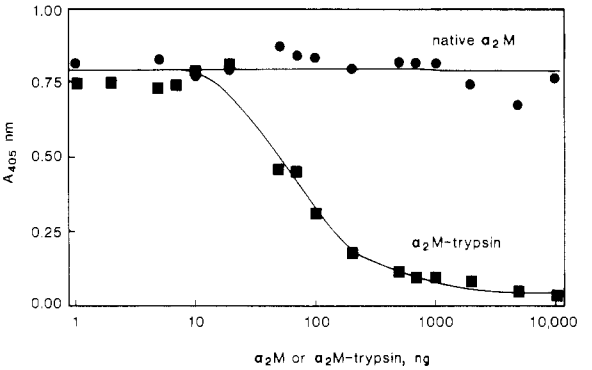

FIGURE 1: Specificity of monoclonal antibody 7H11D6 determined by a competitive binding assay. Wells of a microtiter plate were coated with 200 ng of α_2M reacted with methylamine. An aliquot (200 μL) of dilutions of native α_2M or α_2M reacted with trypsin was preincubated with 6 ng of 7H11D6 for 1 h prior to addition to the microtiter plate. Following incubation and washing, bound antibody was detected by using a rabbit anti-mouse IgG–alkaline phosphatase conjugate.

RESULTS

Specificity of Monoclonal Antibody 7H11D6 for α_2M Reacted with Proteinases or with Methylamine. Spleen cells, obtained from mice immunized with methylamine-treated α_2M, were fused with the mouse myeloma cell line P3-X63-Ag8.653. From this fusion, several cells producing antibodies to α_2M were identified by using a solid-phase ELISA assay, in which wells of microtiter plates were coated with α_2M reacted either with trypsin or with methylamine. By use of a competitive binding assay (see below), one of the cell lines obtained from this fusion was found to secrete IgG specific for α_2M–proteinase complexes and for methylamine-treated α_2M. This cell line was cloned twice, with each cloning performed on wells producing antibody which were at average dilutions of one cell or less per well and which visibly demonstrated growth as a single clone of cells. The antibody was purified by affinity chromatography, and a single band of approximately 150 kDa was observed upon SDS–PAGE under nonreducing conditions. Analysis of the purified antibody by SDS–PAGE under reducing conditions revealed that the molecule is comprised of two subunits, with approximate molecular weights of 50K and 25K, which is characteristic of the heavy and light chains of IgG. The purified antibody was subtyped by the Ouchterlony technique and identified as belonging to an IgG_1 subclass with a κ light chain.

The specificity of the cloned antibody for α_2M–proteinase complexes was examined by using a competitive binding assay (Figure 1). This assay measures the ability of the α_2M–trypsin complex to compete with binding of the antibody to methylamine-treated α_2M previously bound to a solid-phase surface. The results shown in Figure 1 represent the average of triplicate determinations and demonstrate the remarkable specificity of this antibody for the α_2M–trypsin complex. No evidence for binding of this antibody to native α_2M or to free trypsin or any other proteinases was apparent in these and other studies. Several other α_2M–proteinase complexes, including the plasmin, chymotrypsin, elastase, and papain, gave essentially identical results (data not shown). The affinity of the antibody for α_2M–proteinase complexes is at least 2–3 orders of magnitude greater than the affinity for native α_2M. Several lines of evidence suggest that the monoclonal antibody is specific for a neoantigen expressed following reaction of α_2M with proteinases or methylamine, and not for a region of the proteinase. In the first place, the antibody binds to methylamine-treated α_2M, an α_2M derivative in which proteinases

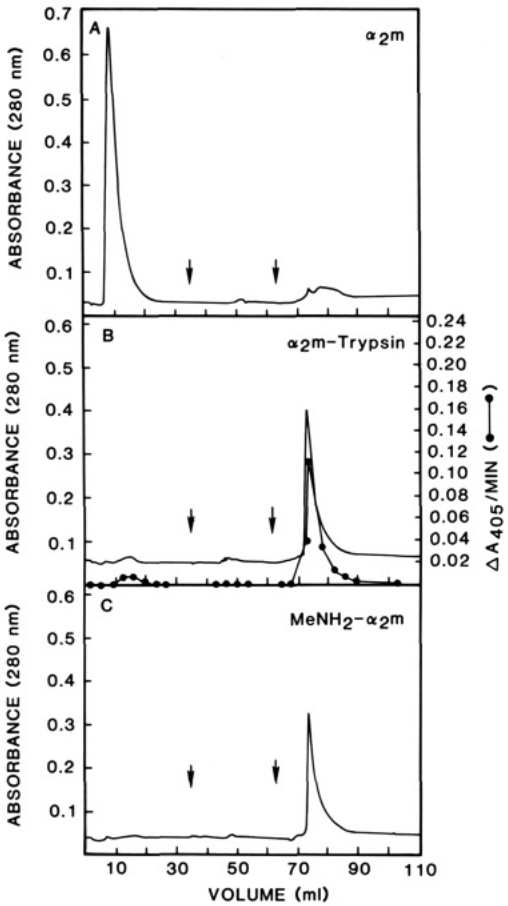

FIGURE 2: Immunoaffinity chromatography of native α_2M (A), α_2M–trypsin complex (B), and α_2M reacted with methylamine (C). The column (1.0 × 16 cm) was equilibrated with 50 mM sodium phosphate/0.5 M NaCl, pH 7.4, and the flow rate was 0.25 mL/min. At the first arrow, the buffer was changed to 50 mM sodium acetate, pH 5.0, and at the second arrow, the buffer was changed to 50 mM sodium acetate, pH 5.0, containing 0.5 M NaCl. In the case of native α_2M, α_2M–trypsin, and α_2M reacted with methylamine, 3.9, 1.9, and 1.9 mg were applied to the column, respectively. When the α_2M–trypsin complex was applied to the column (B), aliquots were removed and assayed for trypsin by using the chromogenic substrate Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (S-2222).

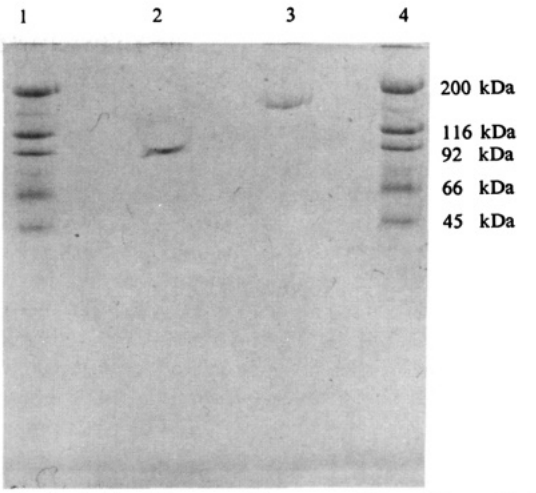

FIGURE 3: Analysis of the material recovered from Figure 2A,B by SDS–PAGE under reducing conditions. Samples were incubated with SDS incubation buffer, and 0.2 μg was applied to a Pharmacia PhastSystem electrophoresis apparatus. Lanes 1 and 4 represent standards which include myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, and ovalbumin. Lane 2, α_2M–trypsin complex recovered from immunoaffinity column (Figure 2B); lane 3, native α_2M recovered from Figure 2A.

are absent. Second, all α_2M–proteinase complexes examined were essentially identical in their ability to compete with the binding of the antibody to methylamine-treated α_2M on a solid-phase surface.

Immunoaffinity Chromatography. The specificity of the antibody was further examined by preparing an affinity column in which the purified antibody was coupled to Affi-Gel 10. Initial experiments measured the binding of α_2M to the affinity column (Figure 2A). The material placed over the column was determined to be greater than 95% active by using the assay described by Steiner et al. (1985) and by examining the preparation by SDS and native PAGE. The majority of the protein applied was not bound to the column, and analysis of this pool by SDS–PAGE under reducing conditions (Figure 3, lane 3) revealed a single 180-kDa band, representing the intact α_2M subunit. Further, examination of the mobility of the fraction upon native PAGE (data not shown) revealed that this unabsorbed fraction migrated as "slow" α_2M, which is characteristic of the mobility of the native molecule (Barrett et al., 1979).

This result contrasts sharply with what happens when native α_2M is reacted with a 1.9-fold molar excess of trypsin prior to immunoaffinity chromatography. The majority of the protein applied to the affinity resin remained bound to the column (Figure 2B). A small amount of trypsin activity was detected in the unabsorbed fractions and accounted for approximately 3–4% of the total activity applied to the column. After the column was washed with equilibration buffer, the buffer was changed to 50 mM acetate, pH 5.0 (first arrow, Figure 2). Elution of the α_2M–trypsin complex from the column was achieved by raising the ionic strength with a buffer of 50 mM acetate, pH 5.0, containing 0.5 M NaCl (second arrow, Figure 2). The protein and trypsin activity coeluted as a single peak, and an 85% recovery of both trypsin activity and protein was obtained. Examination of the protein eluted from the column by SDS–PAGE under reducing conditions (Figure 3, lane 2) revealed that all of the α_2M subunits had been cleaved into 85–90-kDa polypeptides. Examination of the eluted fraction by native PAGE revealed that the fraction migrated in the "fast" position (data not shown), which is characteristic of α_2M–proteinase complexes and of α_2M reacted with methylamine (Barrett et al., 1979). Examination of the binding of a number of α_2M–proteinase complexes to the affinity resin indicated that the interaction of complexes with immobilized antibody binding is independent of the proteinase used to form the complex and that the elution conditions were identical for all complexes examined thus far. The yield of α_2M–proteinase complexes from the column typically ranged from 70% to 85%. The capacity of the column was examined by applying a large excess of α_2M–proteinase complex, washing the column extensively, and quantitating the amount of complex that could be eluted from the column. The capacity of the column, determined in this manner, was 0.6 mg of α_2M–proteinase complex/mL of resin and also appeared to be independent of the type of complex used.

Methylamine inactivates α_2M by cleaving the thiol ester bonds present in the molecule (Van Leuven et al., 1982), resulting in a conformational change (Gonias et al., 1982; Björk & Fish, 1982; Straight & McKee, 1982). Therefore, native α_2M was reacted with 200 mM methylamine for sufficient time to ensure that the reaction was finished as judged by a completion of the alterations in TNS fluorescence (Strickland & Bhattacharya, 1984). This material bound to the affinity resin (Figure 2C) and was eluted by using conditions identical with those required to elute α_2M–proteinase

complexes. In the experiments shown in Figure 2, a two-step elution procedure was used, since it was found that this procedure (i.e., first lowering the pH and then increasing the ionic strength) resulted in sharpened peaks.

Preparation and Isolation of 1:1 Binary α_2M–Proteinase Complexes. Pochon and Bieth (1982) presented evidence suggesting that the two α_2M proteinase binding sites are identical and independent. By making this assumption, it is possible to calculate the distribution of proteinase between binary (1:1) and ternary (1:2) complexes at various mole ratios of the inhibitor to enzyme. The results of these calculations predict that at high molar ratios of inhibitor to proteinase, most of the complexes formed exist as binary complexes. In all of the experiments in this study, the mole ratio of inhibitor to proteinase was ≥5, under which conditions ≥95% of the complexes formed are binary complexes. Binary complexes of trypsin, plasmin, and thrombin were all prepared by reacting the respective enzyme with large amounts of inhibitor and separating the α_2M–proteinase complex from native α_2M by immunoaffinity chromatography. Despite the large excess of native α_2M present, the ability of the immobilized antibody to specifically recognize α_2M–proteinase complexes was retained, and all of the trypsin activity was associated with the material eluted from the immunoaffinity column. The peak was pooled, and the recovery of protein and trypsin activity was determined to be 70% of that expected for a 1:1 complex calculated from the active trypsin concentration used in the initial reaction to prepare the complex.

Several control experiments were performed to ensure that the conditions used for elution of the complexes from the affinity resin do not alter their properties. In the first series of experiments, equimolar amounts of either trypsin or thrombin were incubated with α_2M to produce complexes. The samples were then split into two aliquots; one was chromatographed on the affinity column while the other was maintained at pH 7.4 as a control. Following elution of the complex from the column, the pH of the sample was readjusted to 7.4, and the properties of the affinity-purified complex were compared with those of the control sample. These studies revealed that no detectable changes in electrophoretic mobility, sulfhydryl content, or TNS fluorescence enhancement occurred in either of these complexes upon immunoaffinity chromatography.

A second control experiment was designed to determine if dissociation of proteinases occurred at pH 5.0, the pH of the elution buffer. Purified binary α_2M–trypsin complex and the initial reaction mixture were subjected to size exclusion chromatography on a Sephadex G-100 column equilibrated with 50 mM sodium acetate/150 mM NaCl, pH 5.0. An aliquot from each fraction was adjusted to pH 7.4 and assayed for trypsin activity by using the synthetic substrate S-2222. In both cases, trypsin activity was exclusively associated with α_2M, and no free trypsin activity was detected. Thus, trypsin does not dissociate from the complex at pH 5.0. These results are consistent with those of Hayse & Harpel (1979), who reported that no dissociation of trypsin from the α_2M–trypsin complex occurs when the complex is exposed to acidic conditions (pH 3.9).

Measurement of the Amount of Proteinase Incorporated into the Purified Binary Complexes. In order to validate that binary complexes are indeed produced during the incubation period, the number of proteinase molecules incorporated per α_2M molecule was determined for each complex isolated. Since the enzyme in the α_2M–proteinase complex still retains activity toward small synthetic substrates, it is possible to measure the amount of enzyme present by monitoring the activity of the proteinase in the complex toward low molecular weight chromogenic substrates. The results of this analysis, summarized in Table I, indicate that all isolated complexes contained less than 1 mol of proteinase/mol of inhibitor. In these and several other experiments, the amount of enzyme incorporated into the complex never exceeded 1 mol of proteinase/mol of α_2M, indicating that indeed binary complexes were produced during the reaction. The overall recovery of α_2M–proteinase complexes was determined by assuming that all of the enzyme associates with α_2M to form a 1:1 complex. Measurements of enzyme activity in the unabsorbed fraction confirmed that this fraction contains very little free proteinase and greater than 97% of the proteinases were associated with α_2M. Since α_2M has a molecular weight at least 10-fold greater than the proteinases used, it has also been assumed for these calculations that the molar extinction coefficient for α_2M–proteinase complexes at 280 nm is identical with that of native α_2M (i.e., the presence of proteinase does not contribute significantly to the absorbance of the complex).

Table I: Recovery and Stoichiometry of Binary α_2M–Proteinase Complexes Purified by Immunoaffinity Chromatography

enzyme	[α_2M] (μM)	[proteinase] (μM)	% recovery[a] of complex	mol of enzyme/mol of α_2M
trypsin	1.57	0.31	70	0.9[b]
plasmin	3.10	0.62	85	0.8[b]
thrombin	2.70	0.54	106	0.6[b]
thrombin	2.70	0.54	124	0.8[c]

[a] Recovery is determined by dividing the total protein recovered (micromoles) from the column by the amount of active enzyme (micromoles) used to form the complex. This assumes that all complexes formed are 1:1 complexes. [b] Determined by measuring the cleavage of chromogenic substrates. [c] Determined by measuring incorporation of ^{125}I-thrombin into the complex.

In several experiments, the purified α_2M–trypsin complex contained approximately 0.7–0.9 mol of trypsin/mol of α_2M, and this mole ratio was unaltered by varying the concentration of the initial reactants. In the case of thrombin and plasmin, values of 0.6–0.8 mol of enzyme/mol of α_2M were obtained from activity measurements. To confirm that activity measurements reflect the amount of enzyme present in the complex, ^{125}I-thrombin was incubated with α_2M and the complex isolated by immunoaffinity chromatography. The value of 0.8 mol of thrombin/mol of α_2M obtained from this experiment is in good agreement with the results obtained from activity measurements. These stoichiometric values slightly lower than 1.0 could result from systematic errors in the procedures used or, more likely, could indicate that some cleavage of α_2M occurs without "trapping" the enzyme. This "nonproductive" cleavage of α_2M has been previously reported by Björk (1984), who observed that immobilized trypsin cleaved the proteinase binding sites of α_2M in a normal manner despite the fact that trypsin was not bound, and by Steiner et al. (1987), who found that plasmin cleaved all of the α_2M subunits despite the fact that only 1.3 mol of enzyme was bound per mole of inhibitor. Any nonproductive cleavage would tend to lower the mole ratio of proteinase associated with α_2M and would increase the yield of the complex obtained in these experiments.

Extent of Subunit Cleavage in the Isolated Complexes. While α_2M appears capable of binding 2 mol of trypsin per α_2M molecule, the stoichiometry of subunit cleavage, thiol ester hydrolysis, and conformational change remain to be established. In order to investigate the extent of subunit cleavage, the purified complexes were analyzed by SDS–

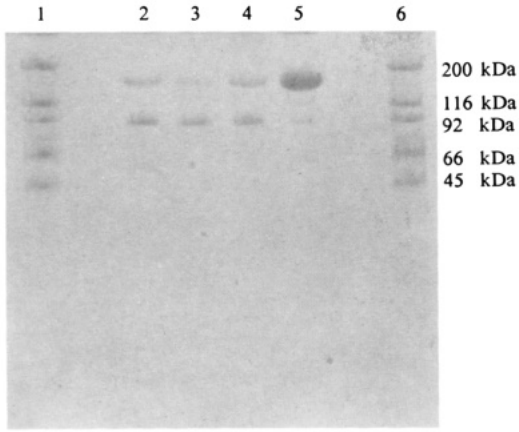

FIGURE 4: Analysis of purified binary α_2M–proteinase complexes by SDS–PAGE under reducing conditions. Samples were incubated with SDS incubation buffer, and 0.2 μg of each complex was applied to a Pharmacia PhastSystem electrophoresis apparatus. Lanes 1 and 6 represent standards which include myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, and ovalbumin. Lane 2, α_2M–thrombin; lane 3, α_2M–trypsin; lane 4, α_2M–plasmin; lane 5, native α_2M.

Table II: Sulfhydryl Content of Purified α_2M–Proteinase Binary Complexes

	mol of SH/mol of α_2M	
complex	initial complex[a]	complex following addition of trypsin or methylamine[b]
α_2M–trypsin	2.6	3.6
α_2M–plasmin	1.9	3.2
α_3M–thrombin	1.8	3.6

[a] Determined by titration of free sulfhydryl groups with *N*-(1-pyrenyl)maleimide. [b] Sulfhydryl groups were titrated with *N*-(1-pyrenyl)-maleimide following reaction of the complex with a 2-fold molar excess of trypsin, or with 200 mM methylamine.

PAGE under reducing conditions, and the results are shown in Figure 4. For these experiments, proteinase activity was inhibited by the addition of DFP prior to adding SDS sample buffer to prevent any nonspecific cleavage of the denatured α_2M. It is apparent that all complexes contain intact subunits, since all samples contain a band that comigrates with the M_r 180 000 α_2M subunit. In the cases of the α_2M–thrombin, α_2M–plasmin, and α_2M–trypsin complexes, approximately 50%, 60%, and 75% of the subunits are cleaved, respectively. Incubation of these α_2M–proteinase complexes with additional trypsin resulted in complete cleavage of all subunits as evidenced by the disappearance of the M_r 180 000 band (data not shown). Although not readily apparent from the gels shown in Figure 4, all complexes contained small amounts of higher molecular weight components similar to those described by Wang et al. (1984).

Measurement of the Number of Thiol Groups Present in Purified Binary Complexes. In the present study, the liberated sulfhydryl groups were titrated either with DTNB or with the fluorescence probe *N*-(1-pyrenyl)maleimide. Virtually identical results were obtained with either method. When *N*-(1-pyrenyl)maleimide was used, the number of thiols was measured by constructing a standard curve using a freshly prepared 1:2 α_2M–trypsin complex as a standard. The sulfhydryl content of the standard was determined by DTNB titration. In several instances, the values obtained with *N*-(1-pyrenyl)-maleimide were validated by using DTNB. The results of these studies, summarized in Table II, reveal that all purified binary complexes contain approximately 2 mol of thiol/mol of complex, thus corroborating the earlier titration studies by Björk et al. (1984). The implications of these results are that binary complexes contain two intact thiol ester bonds, and two thiol ester bonds that have been hydrolyzed. Since addition of excess trypsin to the isolated complexes resulted in cleavage of all α_2M subunits, the effect of excess trypsin on the generation of new thiol groups was measured. The results (Table II) demonstrate that in all cases additional sulfhydryl groups appeared following the incubation. Similar results were obtained when the complexes were incubated with methylamine.

Table III: Effect of Initial Concentrations of α_2M and Trypsin on the Properties of the Purified Binary Complexes

			mol of SH/mol of α_2M	
[α_2M] (μM)	[trypsin] (μM)	% subunits[a] cleaved	initial complex	complex plus trypsin
0.19	0.04	60–75	2.0	3.2
1.57	0.31	60–75	2.6	3.6
10.6	2.12	60–75	2.0	2.9

[a] Estimated SDS–PAGE of the complex following reduction.

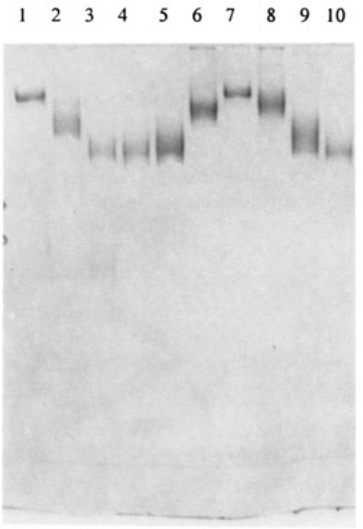

FIGURE 5: Analysis of purified binary α_2M–proteinase complexes by native PAGE. Approximately 10 μg was applied to each well. Lane 1, native α_2M; lane 2, binary α_2M–trypsin complex; lane 3, binary α_2M–trypsin complex reacted with trypsin; lane 4, ternary α_2M–trypsin complex; lane 5, binary α_2M–thrombin complex reacted with trypsin; lane 6, binary α_2M–thrombin complex; lane 7, native α_2M; lane 8, binary α_2M–plasmin complex; lane 9, binary α_2M–plasmin complex reacted with trypsin; lane 10, ternary α_2M–trypsin complex.

Effect of Initial Concentrations on Properties of the Purified Binary Complex. The effect of the initial concentrations of α_2M and trypsin on the properties of the purified complex were examined, since it has been suggested that the stoichiometry of the various reactions might depend upon these parameters (Christensen & Sottrup-Jensen, 1984). For these experiments, the initial concentration of trypsin was varied from 0.04 to 2.12 μM, while maintaining a constant molar ratio of α_2M in each case. After incubation, the complex was isolated, and the properties were measured. The results, summarized in Table III, demonstrate that varying the initial proteinase and inhibitor concentrations over a 50-fold concentration range has very little effect on the extent of subunit cleavage and moles of thiol groups available for titration.

Analysis of Purified Binary Complexes by Nondenaturing PAGE. The reaction of human α_2M with amines or with proteinases has been shown to be accompanied by changes in hydrodynamic volume, evidenced by a higher sedimentation coefficient and an increased mobility in gradient gel electro-

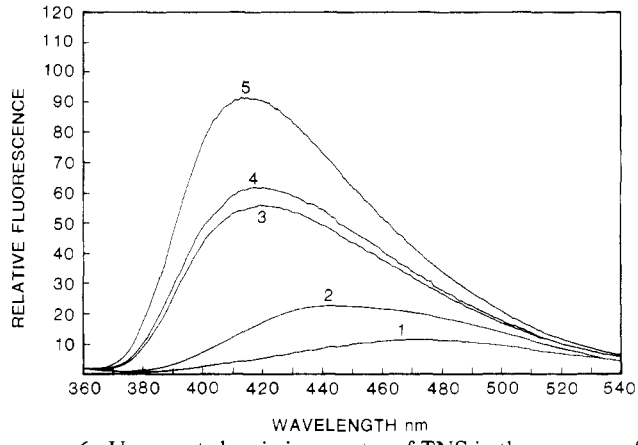

FIGURE 6: Uncorrected emission spectra of TNS in the presence of buffer (1), 0.5 μM α_2M (2), 0.5 μM purified binary α_2M–trypsin complex (3), 0.5 μM purified binary α_2M–trypsin complex reacted with additional 1 μM trypsin (4), and 0.5 μM ternary α_2M–trypsin complex (5). The final concentration of TNS was 50 μM, and all scans were carried out at room temperature. Excitation was at 315 nm, and excitation slits were 2 nm while emission slits were 16 nm.

phoresis (Björk & Fish, 1982; Barrett et al., 1979). The migration behavior of the isolated binary complexes upon nondenaturing gel electrophoresis was examined, and the results are shown in Figure 5. For reference, the migration of native α_2M is shown in lanes 1 and 7, and that of a ternary α_2M–trypsin complex, prepared by reacting α_2M with a 2-fold molar excess of trypsin, is shown in lanes 4 and 10. A comparison of the migration of native α_2M with that of the ternary α_2M–trypsin complex demonstrates the increased mobility characteristic of α_2M–proteinase complexes and α_2M reacted with methylamine (Barrett et al., 1979). The purified binary α_2M–trypsin (lane 2), α_2M–thrombin (lane 6), and α_2M–plasmin (lane 8) complexes all have intermediate mobilities, often with quite broad bands. Reaction of each purified complex with trypsin resulted in an increased mobility (lanes 3, 5, and 9), similar to that observed for the ternary α_2M–trypsin complex. In the case of the α_2M–thrombin (lane 5) and α_2M–plasmin (lane 9) that was subsequently reacted with trypsin, a rather broad band appeared with a mobility close to, but not identical with, that of the ternary complex. The appearance of bands of intermediate mobility has been reported previously for α_2M preparations maintained a 4 °C for prolonged periods (Van Leuven et al., 1981), for α_2M isolated from aged plasma (Nelles et al., 1980), during the reaction of α_2M with methylamine (Larsson et al., 1985), and for the α_2M–plasmin complex (Roche & Pizzo, 1987).

TNS Fluorescence Studies. Previous studies have demonstrated that the fluorescent dye TNS is a sensitive probe of conformational changes occurring in α_2M upon proteolysis or nucleophilic modification of the inhibitor (Strickland & Bhattacharya, 1984; Strickland et al., 1984). Further, the fluorescent properties of the dye are not altered to any great extent in the presence of proteinases alone, rendering this an extremely useful probe of the conformational alterations occurring in the inhibitor. In the present studies, the TNS fluorescence was measured in the presence of native α_2M, purified binary α_2M–proteinase complexes, and α_2M that had reacted with excess trypsin. Representative data for the α_2M–trypsin complex are shown in Figure 6. The fluorescence of TNS in the presence of the binary α_2M–trypsin complex (curve 3) is intermediate between that observed for native α_2M (curve 2) and that measured for the ternary α_2M–trypsin complex (curve 5). In this and all other binary α_2M–proteinase complexes examined, the level of fluorescence was found to

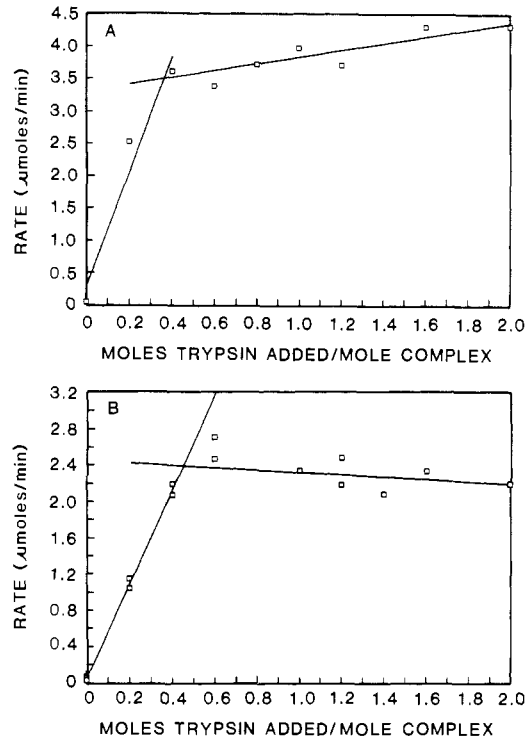

FIGURE 7: Titration of the purified α_2M–plasmin (A) and α_2M–trypsin (B) complex with trypsin. Affinity-purified α_2M–plasmin and α_2M–trypsin binary complexes, prepared as described under Methods, were treated with DFP to inhibit proteinase activity prior to the titration. Titrations were carried out in 50 mM Tris/150 mM NaCl, pH 7.4. The concentration of α_2M–plasmin was 0.073 μM, while the concentration of α_2M–trypsin was 0.079 μM. The appropriate amounts of trypsin were added, and following a 30-min incubation period, free trypsin was inhibited by the addition of 2 μM SBTI. Bound trypsin activity was measured by using the chromogenic substrate Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (S-2222).

lie between that observed for native α_2M and that observed for the ternary α_2M–trypsin complex. In the range of protein concentrations utilized, the fluorescence signal is directly dependent upon α_2M–proteinase concentration. The implications of these data are that in all binary α_2M–proteinase complexes examined, the conformational changes appear incomplete. Reaction of the α_2M–trypsin complex with additional trypsin (curve 4) or methylamine (data not shown) results in only a slight increase in the fluorescence of the dye. Similar results were obtained with the α_2M–plasmin and α_2M–thrombin binary complexes. At this time, the reason for this is unknown but does reinforce the observations that substantial differences exist in the properties of α_2M–proteinase complexes depending upon the initial reaction conditions (Steiner et al., 1987; Christensen & Sottrup-Jensen, 1984).

Binding of Additional Proteinase by Purified Binary α_2M–Proteinase Complexes. The ability of purified binary α_2M–proteinase complexes to bind a second mole of proteinase was examined. For these studies, binary α_2M–trypsin and α_2M–plasmin complexes were prepared and purified, and proteinase activity was inhibited by treatment of the complex with 10 mM DFP. Following dialysis, the complex was titrated with trypsin, and the amount of additional proteinase bound was determined by measuring the activity of the enzyme following incubation of the complex with SBTI. The results of these experiments, shown in Figure 7, demonstrate that the α_2M–plasmin complex is capable of binding 0.3 mol of trypsin/mol (Figure 7A), while the α_2M–trypsin complex binds 0.4 mol of trypsin/mol of complex (Figure 7B). Since SBTI is capable of binding to trypsin in the α_2M–trypsin complex (Bieth, 1981), an additional experiment was carried out. The purified α_2M–trypsin

binary complex, in which trypsin activity was inhibited with DFP, was incubated with a 2-fold molar excess of trypsin, and the complex was purified by immunoaffinity chromatography to separate free from bound trypsin. The amount of trypsin associated with the complex was determined by measuring the hydrolysis of S-2222. The results of this experiment indicate that 0.4 mol of additional trypsin was bound per mole of binary complex, in agreement with the data obtained from titration studies.

Discussion

The reaction of proteinases with inhibitors generally results in conformational changes in the inhibitor. These changes can be detected by immunochemical techniques since neoantigens are generated on the inhibitor molecule and have been detected when antithrombin III reacts with thrombin (Collen & deCock, 1978), when α_2-antiplasmin reacts with plasmin (Plow et al., 1980), and when C1 inhibitor reacts with kallikrein (deAgostine et al., 1985). Marynen et al. (1981) were the first to obtain evidence that neoantigens are generated when α_2M reacts either with proteinases or with methylamine. In the present study, a monoclonal antibody was obtained that does not bind to native α_2M but is specific for a neoantigen that is generated when α_2M reacts with proteinases or with methylamine. The specificity of the antibody has been demonstrated in a competitive ELISA assay and by examining the binding of α_2M derivatives to immobilized antibody. These studies revealed that fully active native α_2M [i.e., α_2M with no subunits cleaved, with 100% activity determined as described by Steiner et al. (1985), and all migrating as the "slow" form upon rate electrophoresis] did not bind to the antibody. However, reaction of native α_2M either with methylamine or with proteinases formed derivatives that were recognized by the antibody.

Once immobilized, the antibody can be used to purify α_2M–proteinase complexes from mixtures containing small amounts of complex and relatively large amounts of native α_2M. The mild conditions required to elute α_2M–proteinase complexes from the affinity column are similar to those employed when purifying native α_2M from plasma and do not result in any detectable alteration in the molecule (Howell et al., 1983). Two methods have been described in the literature for separating proteinase complexes from native α_2M. Van Leuven et al. (1985) described a technique utilizing a TSK Phenyl-5PW HPLC column that separates the modified forms from the native molecule, while Gonias et al. (1986) have described the partial separation of α_2M variants by HPLC on a TSK-G4000 SW size-exclusion column. In the latter case, the column permitted partial resolution of binary α_2M–trypsin from ternary α_2M–trypsin complexes. While these techniques appear to be promising tools for the rapid separation of α_2M variants, they are not feasible for the purification of complexes present in biological fluids.

The availability of a method for separating small amounts of α_2M–proteinase complex from large amounts of native α_2M has permitted purification of binary α_2M–proteinase complexes whose subsequent characterization has clarified some details of the proteinase reaction with this inhibitor. The isolated complexes contained approximately two intact thiol ester bonds, and the conformational change was incomplete when compared with complexes formed in the presence of excess proteinases. The extent of subunit cleavage varied between 50% and 75%, depending upon the enzyme used to form the complex. It is possible that some of this subunit cleavage occurs following formation of the complex mediated by the bound enzyme. Such cleavage of the complex by bound enzyme has been reported for the α_2M–plasmin complex (Roche & Pizzo, 1987). Overall, these data are consistent with the concept that α_2M contains two functional units and that the association of 1 mol of enzyme with α_2M largely results in alterations in one functional unit. This concept is consistent with the observations of Gonias and Pizzo (1983b) and Liu et al. (1987), who have successfully isolated functional α_2M "half-molecules" by limited reduction or by treatment of α_2M with 3 M urea, respectively.

The observation that binary α_2M–proteinase complexes, formed under conditions of inhibitor excess, contain alterations primarily in one of two functional units suggests that these complexes might be capable of binding additional proteinases. Binding studies indicated that the binary complexes of trypsin and plasmin were both able to bind some additional trypsin. In either case, however, the complex bound less than 1 mol of enzyme/mol of complex. Since it is possible to incorporate 2 mol of trypsin per α_2M molecule when the inhibitor reacts with excess enzyme, the binding of 0.4 mol of trypsin/mol by the binary complex suggests that some rearrangement of the complex is occurring, reducing the ability of the complex to bind additional proteinases.

One of the puzzling aspects of α_2M chemistry is the observation that the binding of proteinases with this inhibitor occurs with varied stoichiometry and seems to depend both on the particular proteinase and on the conditions of the reaction (Christensen & Sottrup-Jensen, 1984). It has been speculated that the size of the proteinase is important for determining the final stoichiometry (Pochon et al., 1978); however, this hypothesis does not adequately explain the experimental observations that the binding stoichiometry of α_2M and trypsin, for example, can vary depending upon the initial conditions of the reaction (Straight & McKee, 1984). A correlation between the rate at which a proteinase is inhibited and the stoichiometry has been noted, and it has been proposed that the association rate of the proteinase with α_2M is important in determining the binding ratio (Straight & McKee, 1984; Howell et al., 1983). The results of the present studies suggest that rearrangement of the complex following association of an enzyme molecule with the inhibitor is one key mechanism by which the binding stoichiometry varies with different enzymes. A clear understanding of this phenomenon is necessary in order to gain insight into the sequence of events that ultimately lead to proteinase inhibition, and future work will be directed toward understanding this characteristic of the α_2M–proteinase reaction.

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