Characterization of Human α_2-Macroglobulin Monomers Obtained by Reduction with Dithiothreitol†

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ABSTRACT: We compared the physicochemical characteristics of α_2-macroglobulin (α_2M) monomers produced by limited reduction and carboxamidomethylation to those of the naturally occurring monomeric α-macroglobulin homologue rat α_1-inhibitor 3 (α_1I_3). Unlike α_1I_3, α_2M monomers fail to inhibit proteolysis of the high molecular weight substrate hide powder azure by trypsin. In contrast to α_1I_3, which remains monomeric after reacting with proteinase, α_2M monomers reassociate to higher molecular weight species (dimers, trimers, and tetramers) after reacting with proteinase. Reaction of α_2M monomers at molar ratios of proteinase to α_2M monomers as low as 0.3:1 leads to extensive reassociation and is accompanied by complete bait-region and thiolester bond cleavage. During the reaction of α_2M monomers with proteinases, the proteinase binds to the reassociating α_2M subunits but is not inhibited. Of significance, all the bound proteinase was covalently linked to the reassociated α_2M species. Treatment of α_2M monomers with methylamine results in thiolester bond cleavage but minimal reassociation. Treatment of α_2M monomers with methylamine followed by proteinase results in complete bait-region cleavage and is accompanied by marked reassociation of α_2M monomers to higher molecular weight species. However, no proteinase is associated with these higher molecular weight forms. We infer that bait-region cleavage is more important than thiolester bond cleavage in driving α_2M monomers to reassociate. Despite many similarities between α_1I_3 and α_2M monomers, significant differences must exist with respect to proteinase orientation within the inhibitor to account for the failure of α_2M monomers to protect large molecular weight substrates from proteolysis by bound proteinase, in contrast to the naturally occurring monomeric homologue rat α_1I_3.

The α-macroglobulins are a group of plasma proteinase inhibitors that are present in all vertebrates and also have been identified in various invertebrates [for review see Sottrup-Jensen (1987)]. α_2M has the broadest specificity of any known proteinase inhibitor, and it is capable of inhibiting proteinases from all four classes: serine proteinases and thiol-, metallo-, and carboxylproteinases (Barrett & Starkey, 1973; Travis & Salvesen, 1983; Sottrup-Jensen, 1987). The superfamily of α-macroglobulins possesses a unique mechanism of proteinase inhibition that distinguishes this group of proteins from other known proteinase inhibitors. Human α_2M inhibits proteinases by sterically "trapping" the enzyme (Barrett & Starkey, 1973). Thus, the proteinase loses its ability to degrade most large molecular weight substrates but retains catalytic activity against small molecular weight substrates (Ganrot, 1966). This trapping results from a conformational change of the molecule following proteolysis of a peptide bond in the area of the molecule called the "bait region" (Barrett & Starkey, 1973; Harpel, 1973; Gonias et al., 1982a). Most, but not all, α-macroglobulins contain a labile thiolester bond (Nagase et al., 1983; Feldman & Pizzo, 1984). During bait-region cleavage, the thiolester bond is cleaved and may form a covalent bond with the proteinase (Sottrup-Jensen et al., 1980); however, the site of covalent bond formation is not at the enzyme's active site (Salvesen & Barrett, 1980; Salvesen et al., 1981). This thiolester bond may also be cleaved by primary amines such as methylamine (Barrett et al., 1979). Incorporation of methylamine into the thiolester of human α_2M results in the inability of the molecule to further bind or inhibit proteinase (Salvesen et al., 1981; Van Leuvan et al., 1981). However, this is not a universal finding; for example, the thiolesters of rat α_2M react with methylamine without a major conformational change. Subsequent treatment with trypsin results in bait-region cleavage and proteinase incorporation into the inhibitor although the proteinase is unable to covalently bind to the subunits (Gonias et al., 1983).

Human α_2M is composed of four identical subunits of M_r = 180 000 that form two disulfide-bonded dimers or half-molecules (Hall & Roberts, 1978; Barrett & Starkey, 1973; Sottrup-Jensen et al., 1983; Gonias & Pizzo, 1983a,b); two dimers are noncovalently associated in the tetrameric, native protein. We proposed that the minimum inhibitory unit of the α-macroglobulin family is a dimer (Gonias & Pizzo, 1983a,b; Feldman et al., 1985). More recently, the monomeric rat protein α_1-inhibitor 3 (α_1I_3) has been extensively characterized (Enghild et al., 1989). This protein consists of a single M_r = 180 000 subunit with considerable sequence homology to human α_2M subunits (Aiello et al., 1988; Braciak et al., 1988). This monomer is a functional proteinase inhibitor only if the proteinase covalently attaches to the inhibitor after thiolester cleavage (Enghild, et al., 1989). Moreover, as with dimeric and tetrameric α-macroglobulin homologues, inhibition by α_1I_3 also involves a steric mechanism. It is unclear what has been the evolutionary force that has driven the functioning unit of the α-macroglobulins to be either dimeric or tetrameric. Because monomeric rat α_1I_3 is an exception to the pattern that dimers are the minimum inhibitory unit, we undertook eval-

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uation of the physicochemical properties of the human α_2M monomer produced by limited reduction. It is demonstrated that these monomers retain proteinase-binding activity but that they are unable to inhibit bound proteinase. These findings are in stark contrast to rat α_1I_3 and to the dimeric and tetrameric α-macroglobulins. It appears that the ability of rat α_1I_3 to inhibit proteinases is a unique feature of this monomeric α-macroglobulin.

Experimental Procedures

Proteins. Native α_2M (Roche et al., 1989), rat α_1-inhibitor 3 (α_1I_3) (Enghild et al., 1989), and α_1-proteinase inhibitor (α_1PI) (Pannell et al., 1974) were purified as previously described. Porcine pancreatic elastase (type IV) (PPE) and bovine trypsin (type VIII) were obtained from Sigma Chemical Co., St. Louis, MO. Human neutrophil elastase (HNE) was a kind gift of Dr. Weislaw Watorek, University of Georgia, Athens, GA. Bovine pancreatic trypsin inhibitor (BPTI), Trasylol, was purchased from Mobay Chemical Corp., Pittsburgh, PA.

Reagents. 3,4-Dichloroisocoumarin (DCI), dithiothreitol (DTT), iodoacetamide (IAA), methylamine, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), *p*-nitrophenyl *p*-guanidinobenzoate (NPGB), and hide powder azure were purchased from Sigma Chemical Co., St. Louis, MO. The fluorometric substrate for PPE and HNE (MeOSuc-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin; suc-AAPV-AMC) was purchased from Enzyme System Products, Livermore, CA. $Na^{125}I$ (17.4 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Electrophoresis reagents and solid-phase lactoperoxidase–glucose oxidase system radioiodination beads were purchased from Bio-Rad, Richmond, CA. Sephadex G-25 was purchased from Pharmacia-LKB, Piscataway, NJ. All other reagents were of the highest quality commercially available.

Active-Site Standardization of Proteins. Trypsin was standardized by active-site titration with NPGB as described by Chase and Shaw (1967). The inhibitory capacity of α_1PI was determined by titration with standard trypsin. α_1PI was then used to standardize HNE and PPE by employing the fluorometric substrate suc-AAPV-AMC. The active concentrations of native α_2M and rat α_1I_3 were determined with standardized trypsin by using hide powder azure as a substrate (Salvesen & Nagase, 1989).

Radiolabeling of Proteinase. Proteinases were labeled with $Na^{125}I$ by the solid-state lactoperoxidase–glucose oxidase system (Bio-Rad, Richmond, CA) according to the manufacturer's recommendations and then desalted on a Sephadex G-25 column. The labelings were performed in 50 mM NaP_i, pH 7.4, and then desalted in the following solutions: dilute HCl, pH 3.0, for trypsin; 50 mM sodium acetate, pH 4.5, and 150 mM NaCl, for HNE and PPE.

Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed on a 5–15% gradient gel (10 cm × 10 cm × 1.5 mm) with the glycine/2-amino-2-methyl-1,3-propanediol/HCl system (Bury, 1981). Samples were incubated in the upper reservoir buffer for 60 min at 37 °C prior to electrophoresis. When samples were reduced, the final concentration of DTT was 60 mM. In samples that contained trypsin, HNE, or PPE, a 5-fold molar excess of BPTI over proteinase was added for 30 min, followed by DCI to a final concentration of 50 μM for 30 min prior to denaturation. Nondenaturing pore-limit gel electrophoresis was performed in 5–20% gradient gels (10 cm × 10 cm × 1.5 mm) in a Tris/EDTA/boric acid buffer system with continuous circulation of upper and lower reservoir buffers at 100 V for 16–18 h (Manwell, 1977). Two to five micrograms of protein was loaded in each gel lane. For any given experiment, the same amount of protein was applied to each lane of the gel. The gels were stained with Coomassie Blue.

Inhibitory Assays. The proteinase inhibitors α_2M, α_1I_3, monomeric α_2M, and α_1I_3 that had undergone limited reduction were compared for their ability to protect trypsin from digesting the high moleculer weight substrate hide powder azure. Increasing concentrations of the proteinase inhibitor (from 0 to 200 nM native α_2M or from 0 to 840 nM α_2M monomer, native α_1I_3, or α_1I_3 that had undergone limited reduction) were reacted with 4.2 nM active-site-titrated trypsin in 0.6 mL of NaP_i, 50 mM, pH 7.4. The reactants were preincubated for 30 min at 23 °C before 0.4 mL of a hide powder azure suspension, 12.5 mg/mL in 0.6 M sucrose and 0.05% Triton X-100, was added. The tubes were agitated at 23 °C to maintain the particulate substrate in suspension. The reaction was terminated by adding 0.4 mL of glycine/HCl, 3 M, pH 3.0. The tubes were centrifuged, and residual proteolytic activity was determined by measuring the A_{595nm} of the supernatant.

Preparation of α_2M Monomers. Solutions of 50 mM DTT and 400 mM IAA were made fresh and used within 60 min. α_2M (2.8 μM) was reacted with 2 mM DTT for 60 min at 23 °C in 50 mM NaP_i, pH 7.4. IAA was then added to the reaction mixture to a final concentration of 10 mM for an additional 30 min. The sample was filtered with a 0.22-μm nylon filter, applied to a 0.5 × 20 cm Mono Q column, and eluted with NaP_i, 50 mM, pH 7.4, with a linear gradient from 40 to 100 mM NaCl (10 mM/mL) by using a Pharmacia FPLC system. A 15-mL wash of start buffer was used prior to protein elution to remove all DTT or IAA. This procedure resolved monomers from dimers. Unless otherwise specified, all monomer experiments were in the eluting NaP_i and NaCl buffer. α_1I_3 was also reacted with 2 mM DTT and 10 mM IAA. The sample was dialyzed against two 1-L changes of NaP_i, 50 mM, pH 7.4, at 4 °C for 6 h prior to characterization. The monomer preparations were then used immediately, since storage of the preparations at 4 °C for several days resulted in extensive reassociation (data not shown).

Absorption of Coefficient of α_2M Monomers. The absorption coefficient A (1%, 1 cm, λ = 280 nm) of native α_2M is 8.93 (Hall & Roberts, 1978). To determine the absorption coefficient of α_2M carboxamidomethylated monomers, the following experiment was performed on five separate preparations. α_2M was radiolabeled with $Na^{125}I$, and α_2M monomers were prepared as described above. The concentration of purified monomers was determined on the basis of radioactivity measurements by using a γ counter (LKB Wallac CliniGamma 1272). Absorption of the purified monomer preparation was measured twice with a split-beam/UV–vis spectrophotometer. The results of the five experiments were averaged to yield an A (1%, 1 cm, λ = 280 nm) of 12.0 with a maximum of 3% variance.

Titration of Thiol Groups. The appearance of thiol groups from native α_2M or α_2M monomers was determined by titration with DTNB (Ellman, 1959). Native α_2M (1–2 μM) or α_2M monomer (2–3 μM) was added to 100 μM DTNB in NaP_i, 50 mM, pH 7.4, and the absorbance was monitored for 3 min. A 3-fold excess of active-site-titrated trypsin was added to the sample, and the appearance of thionitrobenzoate ion was monitored at A_{412nm} in a split-beam/UV–vis recording spectrophotometer with an extinction coefficient at A_{412nm} of 13 600 M^{-1} cm^{-1}. When thiolester bond cleavage due to the reaction with methylamine (50–200 mM) was investigated, the pH of the NaP_i buffer was adjusted to 8.0. The appearance

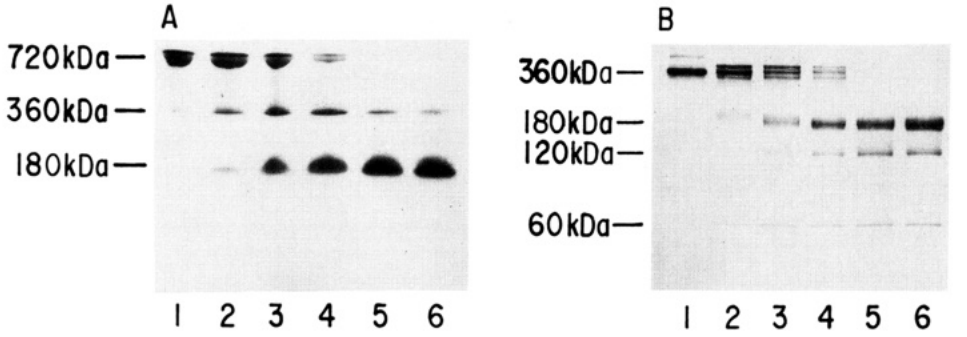

FIGURE 1: Electrophoretic properties of α_2M preparations following reduction and carboxamidomethylation. α_2M was reduced at increasing concentrations of DTT for 60 min as described under Experimental Procedures and subjected to pore-limit gel electrophoresis (5–20%) in panel A or SDS–PAGE (5–15%) without further reduction in panel B. For each panel, the lanes contain α_2M treated with DTT at the following concentrations: lane 1 ,0; lane 2, 0.125 mM; lane 3, 0.25 mM; lane 4, 0.5 mM; lane 5, 1.3 mM; lane 6, 2.0 mM. Not shown are concentrations above 2.0 mM DTT since these are not substantially different from the pattern in lane 6. The bands at 120 and 60 kDa in panel B are the heat-fragmentation products from rupture of the thiolester bonds (Harpel et al., 1979).

of thionitrobenzoate ion was monitored for a minimum of 60 min.

Quantitation of Protein Alkylation. Following reduction and carboxamidomethylation, the extent of modification was determined by completely reducing the derivatized monomeric α_2M (2–3 μM) or α_1I_3 (2 μM) in a solution of 6 M guanidine hydrochloride, 65 mM DTT, and 50 mM Tris-HCl, pH 8.0, at 37 °C for 30 min. DTT was removed by gel filtration chromatography on a Sephadex G-25 column equilibrated with 6 M guanidine hydrochloride and 50 mM Tris-HCl, pH 8.0. The thiol groups were determined by DTNB assay (Ellman, 1959).

Determination of Radioactive Trypsin Bound to α_2M Monomers. To determine the percent of trypsin that was covalently bound versus noncovalently associated with the α_2M species, the following experiment was performed. Varying concentrations of active-site-titrated radiolabeled trypsin were incubated with 10 μg of monomeric α_2M for 5 min at 23 °C. The trypsin was inactivated by the addition of a 5-fold molar excess of BPTI over proteinase and allowed to react for 30 min, followed by the addition of DCI to a final concentration of 50 μM for an additional 30 min. Half of the sample was incubated with nondenaturing pore-limit gel sample buffer at 23 °C for 30 min, and the remaining half of the sample was incubated with nonreducing, denaturing SDS–PAGE sample buffer at 37 °C for 60 min. The samples were loaded and electrophoresed as described above. The gels were strained with Coomassie Blue, destained, placed in swelling solution, and dried, and autoradiograms were performed. Each band from the nondenaturing pore-limit gel (corresponding to the monomer, dimer, trimer, and tetramer) was excised and counted in a γ counter. The radioactivity associated with each band was calculated as a percent of the total radioactivity of the four bands in that lane. To calculate the amount of covalently bound trypsin–α_2M species, the radioactivity associated with bands above 30 000 on the nonreducing SDS–PAGE was cut out and counted. The value for covalently bound proteinase was obtained after dividing by the total radioactivity for that lane.

Results

Preparation and Purification of α_2M Monomers. Evaluation of α_2M that was treated with varying concentrations of DTT (0.25–10 mM) for a varying amount of time (30 and 60 min) by nondenaturing pore-limit gels and nonreducing SDS–PAGE led us to choose 2 mM DTT for 60 min as the best conditions in which to prepare monomers (Figure 1). These conditions resulted in approximately 90% of α_2M migrating as monomers on a nondenaturing pore-limit gel, with the remaining 10% of the protein distributed between dimers (M_r = 360 000) and tetramers (M_r = 720 000). When the sample was electrophoresed in SDS under nonreducing conditions, approximately 90% of the protein migrated at the M_r = 180 000 position, with the remaining 10% at the M_r = 360 000 position. As it was impossible to obtain pure monomers by limited reduction under nondenaturing conditions, we further purified the monomers by ion-exchange chromatography. The monomer eluted at a NaCl concentration between 40 and 100 mM. As determined by nondenaturing pore-limit gel electrophoresis, the monomer preparation was essentially homogeneous after this procedure.

The number of disulfide bonds reduced and carboxamidomethylated was determined as described under Experimental Procedures. For six separate α_2M monomer preparations, 6.0 ± 0.5 disulfide bonds were reduced and carboxamidomethylated out of a total of 12.0 per subunit (Sottrup-Jensen et al., 1983; Jensen & Sottrup-Jensen, 1986). Because the primary purpose was to compare the characteristics of α_2M monomers with the naturally occurring monomeric rat proteinase inhibitor α_1I_3, α_1I_3 was also treated under conditions identical with those in the human α_2M monomer preparation. The number of intrasubunit disulfides that were reduced was determined as described for monomeric α_2M. Four disulfide bonds were reduced and carboxamidomethylated out of a total of 12 predicted disulfides within the α_1I_3 molecule (Aiello et al., 1988; Braciak et al., 1988). While the number of disulfides that were reduced under the experimental conditions differed somewhat between monomeric human α_2M and rat α_1I_3, it is likely that this difference in part reflects the presence of two interchain disulfide bridges per half-molecule in native α_2M (Jensen & Sottrup-Jensen, 1986), which are more easily more easily accessible to the DTT (Gonias & Pizzo, 1983a,b).

Inhibitory Capacity of Monomeric α_2M. The first functional question to be addressed was whether monomeric α_2M functions as a proteinase inhibitor. This was evaluated by determining the ability of monomeric α_2M to protect the large molecular weight substrate, hide powder azure, from proteolysis by trypsin. Three monomeric α_2M preparations failed to show any ability to protect hide powder azure from proteolysis (Figure 2). In comparison, reduced α_1I_3 had virtually the same ability to protect hide powder azure from proteolysis as did native α_1I_3 (Figure 2). These data suggest that reduction and carboxamidomethylation do not in themselves explain the loss of protective ability seen with human α_2M monomers.

Thiolester Bond Cleavage in Monomeric α_2M. We next investigated the thiolester bond of α_2M monomer by DTNB

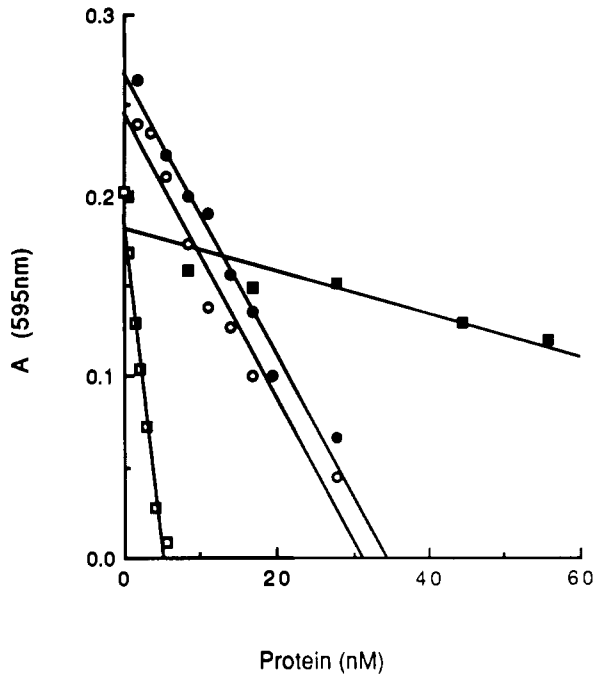

FIGURE 2: Degradation of hide powder azure by trypsin incubated with α-macroglobulin species. Native α_2M (□), α_2M monomers (■), native α_1I_3 (○), and reduced α_1I_3 (●) were treated with trypsin as described under Experimental Procedures. The ability of the α-macroglobulin species to protect the hide powder azure from proteinase digestion was then assessed as previously described (Enghild et al., 1989).

assay. Reaction of the human monomer with trypsin or methylamine resulted in exposure of 0.8 thiols per subunit. Under the same reaction conditions, 3.6 thiols were exposed for the tetrameric human α_2M, or the equivalent of 0.9 thiol per subunit. Thus, the failure of monomeric α_2M to protect hide powder azure from proteolytic cleavage by trypsin cannot be accounted for by a failure of thiolester activation.

Reassociation of α_2M Monomers to Higher Molecular Weight Forms. We have previously demonstrated that human tetrameric α_2M can be dissociated into "half-molecules" by reduction at lower concentrations than those employed to produce monomeric α_2M (Gonias & Pizzo, 1983a,b). These half-molecules reassociated to form tetramers when treated with trypsin (Gonias & Pizzo, 1983a). We next therefore investigated the ability of monomeric α_2M to reassociate to higher molecular weight forms in response to a reaction with proteinases or methylamine.

Varying ratios of radiolabeled, active-site-titrated trypsin were reacted with a constant amount of monomeric α_2M (Figure 3). The nondenaturing pore-limit gel demonstrates the near uniformity of the reassociation of monomers to higher molecular weight forms at trypsin to α_2M molar ratios greater than or equal to 0.3:1.0. The reduced SDS–PAGE (Figure 3) shows that virtually 100% of the bait regions are cleaved at molar ratios greater than or equal to 0.3:1.0 of trypsin to monomeric α_2M. Once all bait regions are cleaved, less than 10% of the monomers remain as nonreassociated subunits. Additionally, a constant ratio of dimer, trimer, and tetramers is found (~40%, ~10%, and ~40%, respectively) regardless of the ratio of proteinase added. Autoradiographs of the pore-limit and reduced SDS gels demonstrate the association of trypsin with the α_2M species. The reactions of monomeric α_2M with radiolabeled trypsin, PPE, and HNE were also compared. While the relative degree of reassociation varies between the different proteinases, all caused monomeric α_2M to reassociate to higher molecular weight forms of M_r = 360000, 540000, and 720000, with the proteinases associating with the α_2M species (Figure 4). While methylamine treatment results in complete thiolester bond cleavage as demonstrated above, only a small fraction of the α_2M monomers reassociates to higher molecular weight forms as shown by the nondenaturing pore-limit gel electrophoresis (data not shown). Addition of trypsin to the methylamine-treated subunits (100 mM) resulted in extensive reassociation without proteinase incorporation.

Nature of Proteinase–Monomer α_2M Interaction. While monomeric α_2M does not protect hide powder azure from proteolysis by trypsin, proteinase reacted with monomers does become covalently associated as described above (Figures 3 and 4). Experiments were designed to determine what fraction of proteinase bound to reassociated human α_2M tetramers is covalently linked to the subunits. These studies (Figure 5) demonstrate that, over a wide range of molar ratios of radiolabeled, active-site-titrated trypsin to monomeric α_2M, all trypsin bound to reassociated α_2M species is via covalent cross-linking to the subunits. Of significant note, the data indicate that, in the reassociated tetramer, it is possible to obtain forms in which one molecule of trypsin is bound to each subunit. As is well-known, native α_2M binds only 1 or 2 mol of proteinase/mol of tetramer (Pochon et al., 1978; Barrett et al., 1979; Swenson & Howard, 1979; Gonias et al., 1982b; Gonias & Pizzo, 1983b; Roche & Pizzo, 1988). Thus, the inability of α_2M monomers to protect hide powder azure from proteolytic cleavage by trypsin is not due to failure of proteinase binding to α_2M species. Rather, these data suggest that reassociated tetramers have properties different from those of native tetramers.

Discussion

The hallmark of the α-macroglobulin superfamily is the ability of the proteinase inhibitor to protect large molecular weight substrates from proteolysis by proteinase (Ganrot, 1966; Barrett & Starkey, 1973). Previous studies by Barrett et al. (1979) demonstrated that α_2M monomers produced by limited reduction reassociated after proteinase treatment but did not prevent proteinases from cleaving hide powder azure. Subsequently, Larsson et al. (1988) employed an enzymatic system to reduce the interchain disulfide bonds of α_2M. These monomers had properties similar to those observed by Barrett et al. (1979). These investigators found that their monomers did not bind ^{125}I-trypsin. By contrast, when we obtained α_2M monomers by chemical reduction with DTT, the individual subunits clearly retained proteinase-binding capacity (Figures 2, 4, and 5) up to a maximal molar ratio of 1:1 (subunit:proteinase). The reason for this difference in results is not apparent, although it is conceivable that the enzymatic system results in some other protein modifications that disrupt proteinase binding. In agreement with these previous reports, however, the monomers demonstrated intact thiolester bonds and reaction with proteinases produced the expected 85- and 95-kDa cleavage products. Treatment with proteinase or methylamine resulted in thiolester bond cleavage.

Our studies demonstrate that human α_2M monomers significantly reassociate to higher molecular weight species after treatment with proteinase. The results of the current study suggest that the conformational change which occurs after bait-region cleavage is particularly important in exposing complementary sites on the monomer that favor reassociation. Additional support for the importance of bait-region cleavage in driving reassociation comes from experiments where α_2M monomers were reacted with methylamine. While complete thiolester bond cleavage occurred, minimal reassociation re-

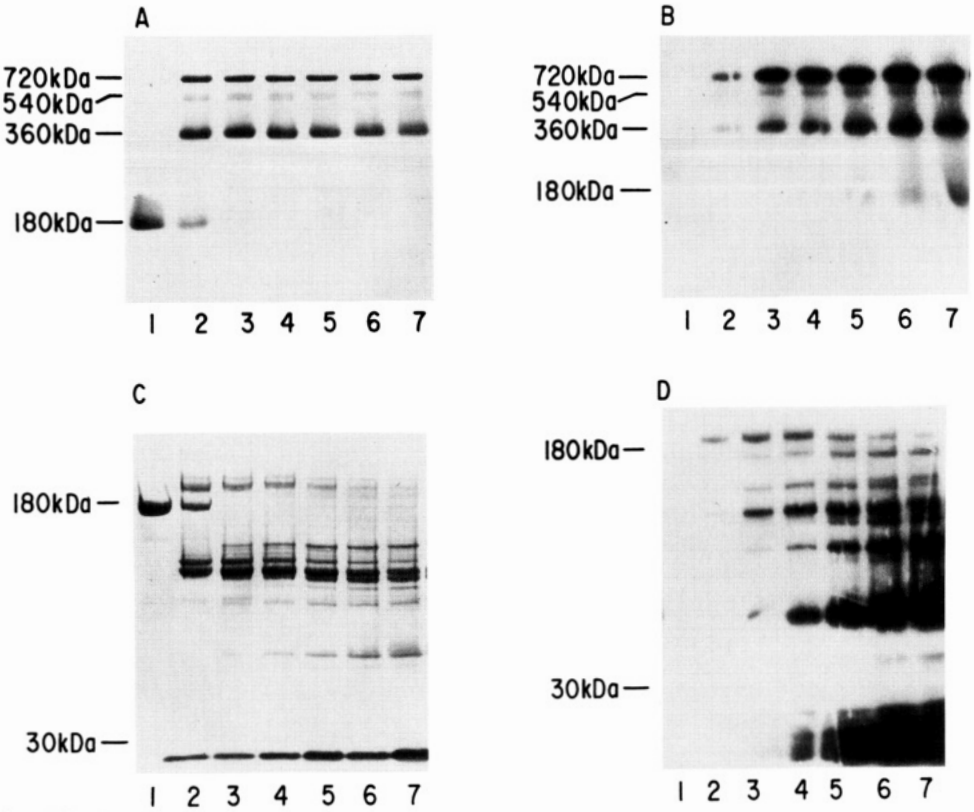

FIGURE 3: Pore-limit and SDS gel electrophoretograms of α_2M monomers reacted with radiolabeled, active-site-titrated trypsin. A constant concentration of α_2M monomer was reacted with increasing molar ratios of the trypsin preparation as described under Experimental Procedures. Panel A is a pore-limit gel (5–20%). Panel B is the autoradiogram of the same gel. Panel C is an SDS–PAGE (5–15%) of the same preparations; the samples were treated with DTT prior to electrophoresis to examine bait-region cleavage. Panel D is an autoradiogram of the gel in panel C that demonstrates the distribution of cross-linked ^{125}I-trypsin. For each gel, the lanes contain incubations in which 1 mol of α_2M monomer is reacted with the following ratios of active-site-titrated ^{125}I-trypsin: lane 1, 0; lane 2, 0.1; lane 3, 0.3; lane 4, 0.5; lane 5, 1.0; lane 6, 2.0; lane 7, 3.0. Panel B demonstrates that the vast majority of the ^{125}I-trypsin is associated with the 360- and 720-kDa species. In panel A, lane 1 shows the homogeneity of the α_2M monomer preparation after limited-reduction and ion-exchange chromatography. (Compare with Figure 1, panel A, lane 6.)

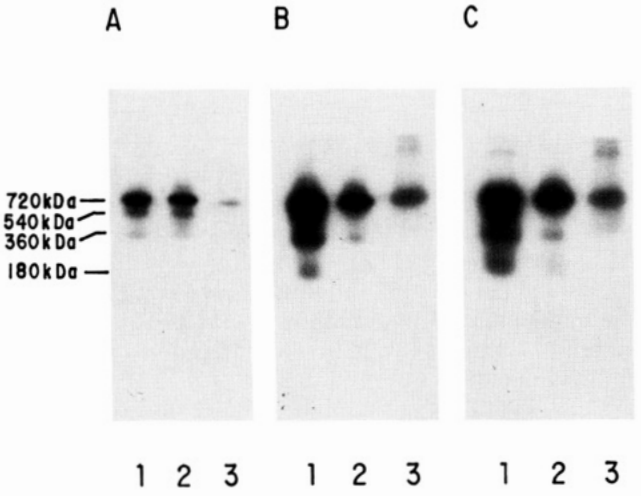

FIGURE 4: Pore-limit gel electrophoretograms of α_2M monomers reacted with radiolabeled, active-site-titrated proteinases. After electrophoresis on a pore-limit gel (5–20%), the gel was subjected to autoradiography as described under Experimental Procedures. The autoradiogram is shown. The legend indicates the position of the α_2M bands seen on the pore-limit gel. The α_2M monomers were reacted with the proteinase at 1:0.1 (α_2M monomer:proteinase) in panel A, 1:1 in panel B, and 1:2 in panel C. The proteinases are the following: lane 1, trypsin; lane 2, PPE; lane 3, HNE.

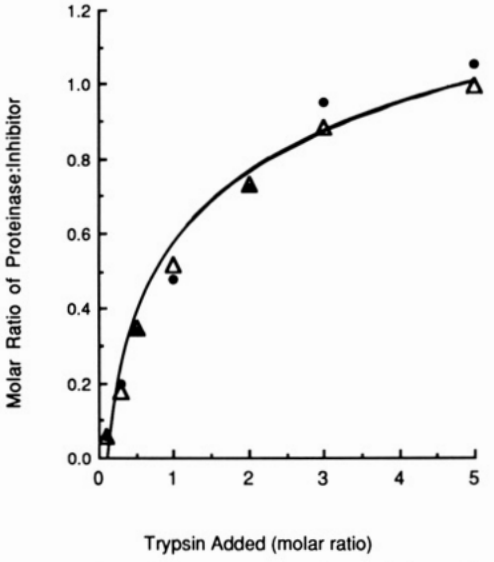

FIGURE 5: Comparison of the extent of total association and covalent association of α_2M monomers treated with active-site-titrated ^{125}I-trypsin. The y axis indicates the extent of trypsin bound per α_2M monomer while the x axis indicates the molar ratio of trypsin:α_2M monomer in the incubation. As described under Experimental Procedures, pore-limit gel electrophoresis was employed to determine the extent of association of ^{125}I-trypsin with the various α_2M species (△). SDS–PAGE of the same samples under nonreducing conditions was employed to determine covalent incorporation (●).

sulted. However, if proteinase was added, a marked increase in reassociation was noted, similar to that seen with primary proteinase treatment. This increase in reassociation was noted despite the fact that virtually all the thiolester bonds had been cleaved prior to the addition of proteinase. In excellent agreement with this hypothesis is the finding that the human dimeric α-macroglobulin homologue pregnancy zone protein

associates to form tetramers when reacted with proteinase but not with methylamine (Christensen et al., 1989). These studies are consistent with our previous studies of the independent and separable nature of the bait region cleavage dependent and thiolester bond cleavage dependent conformational change in α_2M (Roche et al., 1989).

While the present studies suggest that bait-region cleavage is the major driving force for reassociation of monomers to higher molecular weight species, the physical presence of proteinase is not essential for this reassociation. This statement is based on our observations of the stoichiometry of human α_2M monomer reassociation after reaction with proteinase. Molar ratios of proteinase to inhibitor as low as 0.3:1 resulted in complete bait-region cleavage and essentially full reassociation to higher molecular weight species. The stoichiometry data are consistent with the observation that the majority of the reassociated forms lack bound proteinase. Since all associated proteinase was covalently bound, we postulate that the proteinase cleaved the bait region, activated the thiolester bond, and resulted in a proteinase-activated "nascent" form similar to that proposed by Sottrup-Jensen et al. (1981). Because catalytic amounts of trypsin lead to complete bait-region cleavage, trypsin must cleave an α_2M monomer bait region and diffuse away. It is then free to cleave another bait region before eventually forming a cross-link with an activated thiolester bond induced by bait-region cleavage. Hence, the conformational change induced by bait-region cleavage is both necessary and sufficient for reassociation, even in the absence of the proteinase.

The ability of rat α_1I_3 to inhibit proteinases is dependent on the formation of covalent cross-links between the inhibitor and the proteinase (Enghild et al., 1989). However, the current studies with synthetically produced human α_2M monomers, which bind proteinases but do not inhibit them, indicate that the ability to form cross-links with the proteinase is a necessary but insufficient event in shielding proteinases from large substrates. These studies therefore suggest that the rat monomer, α_1I_3, must bind proteinase differently than human α_2M to explain the ability of α_1I_3 to shield the proteinase. It is likely that covalently bound proteinase has a different orientation relative to the inhibitor subunits in the α_1I_3–proteinase complex versus the α_2M–proteinase complex. Such a difference in orientation must shield the proteinase active site from access to large substrates in the α_1I_3–proteinase complex and not the α_2M–proteinase complex. This difference in orientation is magnified when one considers that the α_2M–proteinase complex is virtually all dimeric and tetrameric and, hence, potentially more able to block the proteinase active site.

The α_2M monomer described in this report does not share these inhibitory properties even though it can covalently bind proteinase. By contrast, human α_2M dimers not only bind proteinase but protect the bound proteinase from soybean trypsin inhibitor (Gonias & Pizzo, 1983a,b). While retention of function by α_2M monomers and half-molecules is clearly different, the structural changes that cause α_2M dissociation into monomers or half-molecules remain uncharacterized. It is somewhat surprising that the extent of disulfide bond reduction in the α_2M monomer preparations was not significantly higher than that reported for half-molecules. Differences in the technique used (amino acid analysis versus DTNB titration) may be responsible for this result; however, it is also possible that complete dissociation of α_2M into monomers as opposed to half-molecules depends on a small number of critical disulfides (perhaps one or two). In unpublished studies from this and a collaborating laboratory, we have used FPLC and electron microscopy to analyze α_2M after treatment with different concentrations of DTT.[1] The extent of α_2M dissociation into half-molecules and monomers was clearly dependent on the concentration of reductant, although some proportion of each species could be detected with most DTT concentrations. Half-molecules and monomers were stable when purified from mixed preparations. Therefore, it should be possible to obtain various highly purified preparations of partially reduced and alkylated α_2M for further chemical and structural characterization.

Registry No. Proteinase, 9001-92-7.

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Mechanism of Insulin Incorporation into α_2-Macroglobulin: Implications for the Study of Peptide and Growth Factor Binding†

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ABSTRACT: In recent years, many studies have suggested a direct role for α_2-macroglobulin (α_2M), a plasma proteinase inhibitor, in growth factor regulation. When coincubated in the presence of either trypsin, pancreatic elastase, human neutrophil elastase, or plasmin, ^{125}I-insulin rapidly formed a complex with α_2M which was >80% covalent. The covalent binding was stable to reduction but abolished by competition with β-aminopropionitrile. Neither native α_2M nor α_2M pretreated with proteinase or methylamine incorporated ^{125}I-insulin. Experiments utilizing α_2M cross-linked with *cis*-dichlorodiammineplatinum(II) indicated that ^{125}I-insulin must be present during α_2M conformational change to covalently bind. A maximum stoichiometry of 4 mol of insulin bound per mole of α_2M and the short half-life of the α_2M intermediate capable of covalent incorporation were consistent with thiol ester involvement. Protein sequence analysis of unlabeled insulin–α_2M complexes, together with results of β-aminopropionitrile competition, confirmed that insulin incorporation occurs via the same γ-glutamyl amide linkage responsible for covalent proteinase and methylamine binding to α_2M. Although intact insulin apparently incorporated through its sole lysine residue on the B chain, we found that isolated A chain also bound covalently to α_2M. Phenyl isothiocyanate derivatization of the N-terminus had no effect on A-chain binding, supporting the possibility of heretofore unreported γ-glutamyl ester linkages to α_2M.

The last 5 years have witnessed an explosion in the number of reports implicating a role for α_2-macroglobulin (α_2M)[1] in the regulation of growth factors. Among numerous other studies, investigations on the binding of purified growth factors to α_2M include work on platelet-derived growth factor (Huang et al., 1984), transforming growth factor β (TGF-β) (O'-Connor-McCourt & Wakefield, 1987; Huang et al., 1988; Danielpour & Sporn, 1990; LaMarre et al., 1990), interleukin-1β (Borth & Luger, 1989), interleukin-6 (Matsuda et al., 1989), and basic fibroblast growth factor (Dennis et al., 1989). Taken as a whole, many of these reports present a number of apparent contradictions, due in part to the complexity of α_2M chemistry.

α_2M is a major plasma proteinase inhibitor that operates through a unique mechanism. Proteolytic cleavage in the "bait region" initiates an electrophoretically detectable conformational change in α_2M, which "traps" the proteinase (Barrett & Starkey, 1973; Salvesen & Barrett, 1980; Feldman et al., 1985). Through a somewhat different mechanism, treatment with small primary amines also results in structural compaction and faster migration through native gel systems. By convention, the native unreacted form is referred to as "slow" α_2M (s-α_2M) and the more compact proteinase- or methylamine-treated form as "fast" α_2M (f-α_2M) (Barrett et al., 1979). The proteinase-induced transition is characterized by cleavage of a reactive β-cysteinyl-γ-glutamyl thiol ester present on each of the four α_2M subunits, and by the appearance of receptor recognition sites that lead to rapid clearance of α_2M–proteinase complexes from the circulation (Ohlsson, 1971a,b; Imber & Pizzo, 1981). Methylamine treatment affects a similar conformational change through direct nucleophilic attack on the

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[1] Abbreviations: α_2M, human α_2-macroglobulin; *cis*-DDP, *cis*-dichlorodiammineplatinum(II); DCI, 3,4-dichloroisocoumarin; DDC, diethyldithiocarbamate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HNE, human neutrophil elastase; PPE, porcine pancreatic elastase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBE, tris/boric acid/ethylenediaminetetraacetic acid buffer system; TGF-β, transforming growth factor β.

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