STUDIES ON HUMAN PLASMA α_2-MACROGLOBULIN-ENZYME INTERACTIONS

EVIDENCE FOR PROTEOLYTIC MODIFICATION OF THE SUBUNIT CHAIN STRUCTURE[*][‡]

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The nature of the molecular interactions which occur between circulating proteolytic enzymes and their plasma inhibitors is not fully understood. One such inhibitor, α_2-macroglobulin, appears to participate in the regulation of several circulating enzyme systems, since it forms a complex with plasmin (1–4), thrombin (5–7), and kallikrein (8–10). In addition, α_2-macroglobulin has been found to bind trypsin (11, 12), chymotrypsin (11, 13), elastase (14), papain, cationic aspartate aminotransferase (13), and subtilisin A (15). After the formation of a complex with α_2-macroglobulin the active site of these enzymes is not fully inhibited since the bound enzymes retain their ability to degrade small molecular weight substrates but have diminished proteolytic activity.

In contrast to the paucity of information concerning the mechanisms underlying the action of plasma proteolytic enzyme inhibitors, the interaction of trypsin with soybean trypsin inhibitor as well as a variety of other protein trypsin inhibitors of nonplasma origin has been extensively studied. Tryptic hydrolysis of a single peptide bond located within a disulfide-bridged loop on the inhibitor molecule (16) appears to be a constant feature of the trypsin inhibitor reaction. This observation has led to the concept that protein inhibitors of trypsin may all have a similar susceptible peptide bond and that attack at this site is necessary for complex formation and enzyme inhibition (17).

The potential importance of α_2-macroglobulin in regulating hemostatic and inflammatory reactions and its apparently unique enzyme-binding properties prompted the present studies. The effect of the proteases trypsin, thrombin, plasmin, plasma kallikrein, and chymotrypsin on the covalent structure of α_2-macroglobulin has been examined. The results indicate that complex formation

between these enzymes and α_2-macroglobulin is accompanied by proteolytic modifications in the subunit chain structure of the inhibitor.

Materials and Methods

All chemicals used were reagent grade. Tosyl arginine methyl ester (TAMe)[1] and *N*-α-acetylglycyl-L-lysine methyl ester acetate (AGLMe) were obtained from Cyclo Chemical, Division Travenol Laboratories, Inc., Costa Mesa, Calif. and benzoyl-DL-arginine-*p*-nitro-anilide (BAPNA) from Mann Research Laboratories, Inc., New York. Soybean trypsin inhibitor (grade SI), pancreatic trypsin inhibitor (grade PSIF), lima bean trypsin inhibitor, bovine trypsin (grade TRL), diisopropylfluorophosphate- (DFP) inactivated trypsin and chymotrypsin (grade WCDS) were obtained from Worthington Biochemical Corp., Freehold, N. J. Dithiothreitol (DTT) was obtained from Calbiochem, San Diego, Calif.; sodium dodecyl sulfate (SDS) from Sigma Chemical Co., St. Louis, Mo.; and urokinase from Abbott Laboratories, North Chicago, Ill.

α_2-*macroglobulin* was prepared from human plasma by a modification of previously described methods (8). Venous blood was collected from normal donors into plastic containers to minimize activation of the Hageman factor (Factor XII). One part anticoagulant containing soybean trypsin inhibitor (0.5 mg/ml of 3.8% sodium citrate) was added to nine parts whole blood. The plasma was harvested after centrifugation at 2,000 × g for 15 min at 4°C. After adsorption of the prothrombin complex with barium chloride and barium sulfate, the adsorbed plasma was precipitated with polyethylene glycol (average molecular weight 3,000–4,000) to a final concentration of 12% (Matheson, Coleman, and Bell, East Rutherford, N. J.) (8). The low density lipoproteins were then removed by ultracentrifugation in potassium bromide (Sp g 1.071). The α_2-macroglobulin was further purified by DEAE-cellulose chromatography (DE-52, H. Reeve Angel and Co., Inc., Clifton, N. J.) using a linear sodium chloride gradient at constant pH (starting buffer, Tris-HCl 0.05M, pH 8.0, and the limit buffer containing 0.4M NaCl), gel filtration chromatography (Bio-Gel A-1.5m, and A-5m, Bio-Rad Laboratories, Richmond, Calif.) and preparative Pevicon block electrophoresis (Mercer Chemical Corp., New York) (18). The final α_2-macroglobulin fraction contained no other proteins identifiable by double diffusion analysis using polyvalent rabbit antihuman serum antibody.

α_2-macroglobulin was also purified from plasma which had been incubated 1 h at 37°C with the plasminogen activator, urokinase (500 U/ml plasma). Determination of the concentration of α_2-macroglobulin preparations was performed by radial immunodiffusion (Hyland Immuno-Plate®, Division Travenol Laboratories, Inc., Costa Mesa, Calif.) (4).

Human Enzymes.—Partially purified human plasma kallikrein was prepared as described (8). This material was free of both plasmin and thrombin. Purified human thrombin (Lot H-1, 180 NIH units per vial) was provided by Dr. David Aronson, Bureau of Biologics, Food and Drug Administration, Rockville, Md. Thrombin-free human plasmin (77 caseinolytic units per milliliter), spontaneously activated in 50% glycerol, was obtained from the Michigan Dept. Public Health, Lansing, Mich.

The specific activity of the trypsin preparations used was experimentally determined by titration with soybean trypsin inhibitor (19) using the substrate BAPNA (20). The activity of plasmin preparations was assayed by a standard caseinolytic assay (21); and that of kallikrein by its TAMe esterase activity (1 U of activity equaling that volume of enzyme which hydrolyzed 1 μmol TAMe per min) (8). The functional capacity of α_2-macroglobulin preparations to form a complex with each of these enzymes was assessed by determining the

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[1] *Abbreviations used in this paper:* AGLMe, *N*-α-acetylglycyl-L-lysine methyl ester acetate; BAPNA, benzoyl-DL-arginine-*p*-nitroanilide; DFP, diisopropylfluorophosphate; DTT, dithiothreitol; SDS, sodium dodceyl sulfate; TAMe, tosyl arginine methyl ester.

residual enzymic activity of a mixture of α_2-macroglobulin and enzyme against small molecular weight substrates after the addition of soybean trypsin inhibitor. This test system reflected the presence of enzyme bound in the α_2-macroglobulin-enzyme complex and therefore protected from inhibition by soybean trypsin inhibitor (3). The substrates used were BAPNA for trypsin (20), AGLMe for plasmin (4), and TAMe for kallikrein (8). Since thrombin is not inhibited by soybean trypsin inhibitor (22), the ability of α_2-macroglobulin to complex with this enzyme was determined by its ability to inhibit the fibrinogen clotting activity of thrombin after preincubation with α_2-macroglobulin for 1 h at 37°C (23).

For the present studies, α_2-macroglobulin was incubated with thrombin, plasmin, and kallikrein in concentrations (detailed in Fig. 3 legend) which were completely bound by the inhibitor. Thus, after complex formation, no free enzyme could be identified. In the case of trypsin, the maximum molar binding capacity of the α_2-macroglobulin preparations for the enzyme was experimentally determined as described by Ganrot (24). Each mole of α_2-macroglobulin was found to bind 1.3–1.7 mol of trypsin, a ratio consistent with previously determined values (25, 26). The molar concentrations of the other proteases examined were estimated from their proteolytic activity, their reported molecular weight and maximum specific activity (27–29).

Mixtures of purified α_2-macroglobulin and the various proteolytic enzymes were incubated at 37°C in buffer (Tris-HCl 0.05M, pH 8.0, containing 0.85% sodium chloride and 0.16% sodium citrate) in the concentrations indicated in the figure legends. The reaction was stopped by adding the mixture to an equal volume of a solution containing 10M urea, 1% SDS and 14 mM DTT. After incubation for 45 min at 37°C, the reduced α_2-macroglobulin incubation mixtures were analyzed by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (30). The protein bands were stained with Coomassie brilliant blue. Densitometric scans of the gels were carried out in a Gilford model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), equipped with a linear transport device.

The molecular weight of the α_2-macroglobulin subunit chain and the proteolytic derivatives resulting from interaction with the various enzymes studied was determined by SDS-polyacrylamide gel electrophoresis as previously described (30). The proteins used for molecular weight markers were reduced before electrophoresis. They included phosphorylase A, molecular weight 94,000 (30) from Sigma Chemical Co.; bovine albumin, 68,000 (30) from Schwarz/Mann, Div. Becton, Dickinson, and Co., Orangeburg, N. J.; and ovalbumin, 43,000 (30) from Pharmacia Fine Chemicals Inc., Piscataway, N. J. Human fibrinogen (Aα-chain, 70,900; Bβ-chain, 60,400; and γ-chain, 49,400) (31) was provided by Dr. Michael Mosesson and the heavy chain of myosin (212,000) (32), by Dr. Paul Dreizen, both of the State University of New York, Downstate Medical Center, Brooklyn, N. Y.

RESULTS

Effect of Trypsin on the Subunit Chain Structure of Human Plasma α_2-Macroglobulin (Fig. 1).—After incubation of trypsin and α_2-macroglobulin no alteration in the band pattern was produced by electrophoresis of unreduced samples in SDS-polyacrylamide gels (Fig. 1, gels *A* and *B*). However, a major alteration in the band pattern of α_2-macroglobulin was evident if the samples were reduced before electrophoresis. After reduction of α_2-macroglobulin which had not been reacted with enzyme, one major protein band with an apparent molecular weight of 185,000 (range 182,000–187,000) was identified (Fig. 1, gel *C*). Several minor slowly migrating components remained near the top of the gel suggesting that under the experimental conditions employed, reduction of α_2-

macroglobulin had been incomplete. A new band, molecular weight 85,000 (range 83,000–87,000), was observed after incubation with trypsin for 1 h, and its appearance was accompanied by depletion of the precursor subunit chain (Fig. 1, gel D). Preincubation of trypsin with soybean or pancreatic trypsin inhibitor prevented this trypsin-induced alteration in the primary structure of α_2-macroglobulin. DFP-inactivated trypsin also failed to alter α_2-macroglobulin. A band corresponding to trypsin was not observed, presumably because the amount of enzyme would have been too low to produce a visible protein band (the molecular weight of trypsin being $\frac{1}{30}$ of that of α_2-macroglobulin).
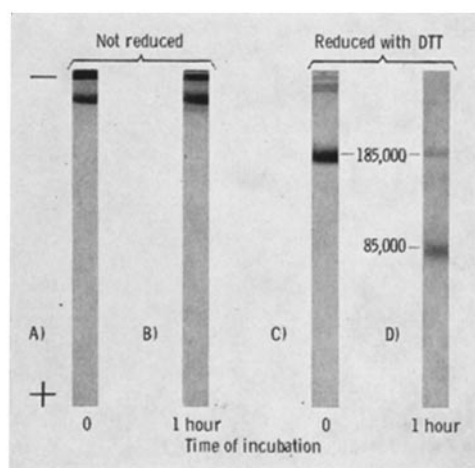

FIG. 1. SDS-polyacrylamide gel electrophoresis (5% gel) of an incubation mixture of an equimolar ratio of human plasma α_2-macroglobulin and trypsin. Equal volumes of α_2-macroglobulin (6.9 mg/ml) and trypsin (195 μg active enzyme per milliliter) were incubated at 37°C. Portions were removed at 0 time and at 1 h and added to an SDS-urea solution (gels A, B) or reduced with a similar solution containing 14 mM dithiothreitol (gels C, D). The approximate molecular weights of the bands that were identified after reduction are indicated.

The Effect of Enzyme Concentration on the Trypsin-Induced Alteration in the Subunit Structure of α_2-Macroglobulin. (Fig. 2).—Trypsin, incubated with α_2-macroglobulin for 1 min before reduction, produced increases in the derivative chain which were proportional to the enzyme concentration (Fig. 2). The derivative chain that formed appeared within 1 min of incubation. The amount formed, as assessed by the densitometric ratio of the precursor to product chain, remained stable for at least an additional 60 min incubation at 37°C. In the molar ratio of 0.15–2.5 mol trypsin relative to 1 mol α_2-macroglobulin, only one derivative band was observed after incubation, suggesting that the parent

chain had been cleaved at, or very near its center.[2] At a trypsin-α₂-macro-globulin molar ratio that approached saturation (1.25) or exceeded the binding capacity of α₂-macroglobulin for the enzyme (2.5), a faster migration of the proteolytic derivative was apparent indicating that further degradation of this component had occurred (Fig. 2).

The formation of a single derivative band appears to be dependent not only on the amount of trypsin but also upon the functional state of α₂-macroglobulin. Acidification of α₂-macroglobulin to pH 2.0 for 30 min at 37°C followed by
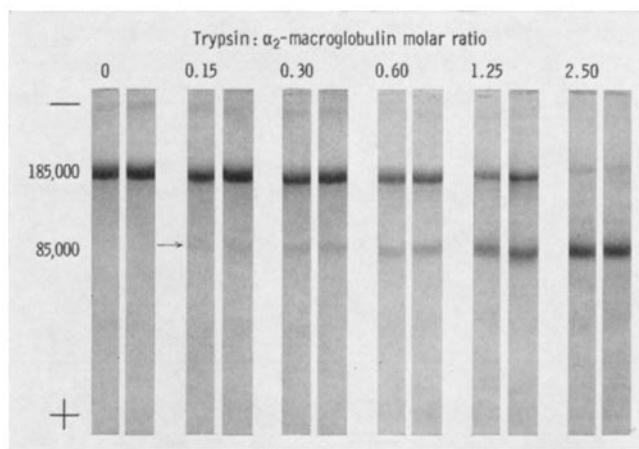

Fig. 2. SDS-polyacrylamide gel electrophoresis (5% gel) after reduction with DTT of incubation mixtures of increasing concentrations of trypsin and a constant concentration of α₂-macroglobulin. Equal volumes of α₂-macroglobulin (4.8 mg/ml) and buffer or serial dilutions of trypsin were incubated 1 and 61 min at 37°C (first and second gel of each pair, respectively), and the reaction stopped by the addition of an SDS-urea-DTT solution. The highest concentration of trypsin was 420 μg/ml, corresponding to a trypsin: α₂-macroglobulin molar ratio of 2.5.

neutralization, destroyed its capacity to form a complex with trypsin, a finding previously reported (26); such treatment, however, produced no evident alteration in its structure as assessed by SDS-polyacrylamide gel electrophoresis of reduced samples. After acid treatment α₂-macroglobulin manifested a greatly increased susceptibility to proteolytic degradation by trypsin since molar ratios of trypsin to α₂-macroglobulin as low as 0.002 degraded α₂-macroglobulin into

[2] There is a discrepancy between the calculated molecular weight of the subunit chain $(2 \times 85,000 = 170,000)$ and that determined by SDS-polyacrylamide gel electrophoresis (e.g., 185,000). This discrepancy may be due to the anomalous migration of some proteins in SDS-polyacrylamide gels (33) or the errors inherent in this method for establishing molecular weight (34). Alternatively, a small molecular weight fragment or fragments whose presence was not appreciated might have been cleaved from the parent subunit chain.

multiple protein bands with molecular weights lower than the 85,000 molecular weight derivative.

Effect of Thrombin, Plasmin, Plasma Kallikrein, and Chymotrypsin on the Subunit Structure of α_2-Macroglobulin (Figs. 3, 4).—Interaction of α_2-macroglobulin with each of these enzymes resulted in the formation of a derivative chain manifesting an electrophoretic mobility identical to the mobility of that derivative chain produced in the α_2-macroglobulin-trypsin interaction. As was the case with trypsin, reduction of α_2-macroglobulin was necessary for the
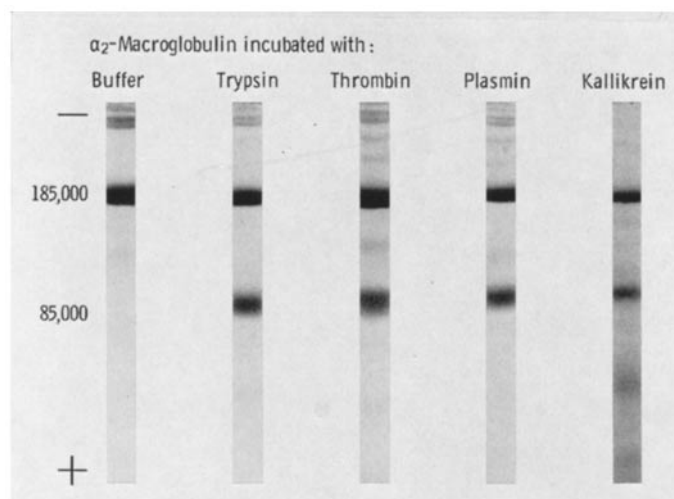

Fig. 3. SDS-polyacrylamide gel electrophoresis (5% gel) after reduction with DTT of α_2-macroglobulin incubated with trypsin, thrombin, plasmin, or plasma kallikrein. These enzymes were incubated with α_2-macroglobulin (165 μg per system) in the following approximate molar ratios: trypsin, 1 (5.4 μg): thrombin, 0.5 (12 NIH units); plasmin, 0.4 (0.15 caseinolytic units); plasma kallikrein, 0.7 (0.14 TAMe esterase units). The reaction was stopped after 1 h at 37°C and the mixture reduced by the addition of an SDS-urea-DTT solution.

demonstration of the derivative band by SDS-polyacrylamide gel electrophoresis (cf. Fig. 1). Small amounts of material with apparent molecular weights greater than 185,000 were present in incubation mixtures of α_2-macroglobulin with thrombin and plasmin that were not apparent in the electrophoretic patterns produced by the individual components. The nature of these bands is not known. Faster migrating material in the α_2-macroglobulin, kallikrein mixture was due to material added with the enzyme preparation itself. To test whether plasmin, a potential contaminant of thrombin and kallikrein preparations, was the active agent, the following experiments were performed. Thrombin was preincubated with soybean trypsin inhibitor and kallikrein with lima bean trypsin inhibitor, substances which inhibit plasmin but not the enzymes

themselves (22, 35). These inhibitors did not prevent the production of the derivative chain indicating that thrombin and kallikrein were the enzymes responsible for the production of the structural change in α_2-macroglobulin.

Chymotrypsin produced a derivative band with a molecular weight similar to that produced by trypsin (Fig. 4). In addition, more faintly staining bands representing two previously unrecognized cleavage products were also formed. Their apparent molecular weights were 90,000 and 75,000, respectively.
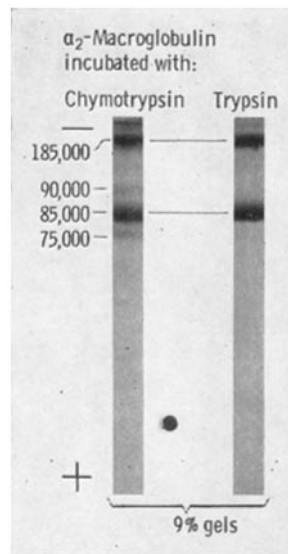

Fig. 4. SDS-polyacrylamide gel electrophoresis (9% gel) after reduction with DTT of α_2-macroglobulin incubated with chymotrypsin or trypsin. An equal volume of α_2-macroglobulin (6.9 mg/ml) was incubated with chymotrypsin (200 μg/ml) or trypsin (195 μg/ml) for 1 h, representing an enzyme to α_2-macroglobulin molar ratio of 1.

Subunit Structure of α_2-Macroglobulin Prepared from Plasma Incubated with Plasminogen Activator, Urokinase (Fig. 5).—The following studies were carried out to determine whether the structural alteration in α_2-macroglobulin induced by incubation with plasmin in purified systems also occurred in a plasma environment. α_2-macroglobulin was isolated from plasma incubated with urokinase (500 U/ml plasma) as described previously (4). SDS-polyacrylamide gel electrophoresis of this preparation, after reduction with dithiothreitol, demonstrated the 85,000 molecular weight derivative chain, whereas the α_2-macroglobulin from a portion of the same plasma not incubated with urokinase showed only the parent chain. Urokinase (300 U/ml), incubated in the absence of plasminogen with purified α_2-macroglobulin (6.9 mg/ml) for 30 min at 37°C failed to produce the derivative chain.

DISCUSSION

This study has shown that the subunit structure of human plasma α_2-macroglobulin is altered by interaction with proteolytic enzymes that it inhibits. α_2-macroglobulin that has not formed a complex with proteases is comprised of subunit chains having a molecular weight of approximately 185,000; with the exception of chymotrypsin, all the proteases studied attack only one region in each subunit chain and produce a single derivative with a molecular weight approximately one-half that of the parent chain. This suggested that the enzyme-susceptible region was located at or near the center of
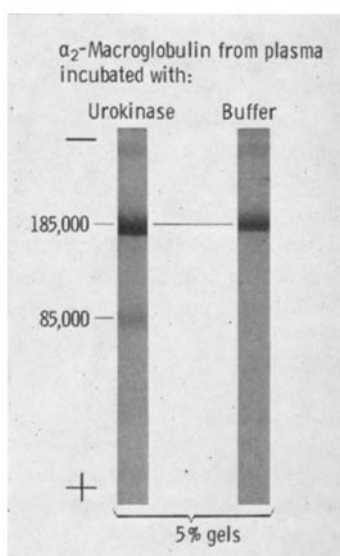

FIG. 5. SDS-polyacrylamide gel electrophoresis (5% gel) after reduction with DTT of α_2-macroglobulin isolated from plasma incubated 1 h with a plasminogen activator, urokinase or isolated from a portion of the same plasma incubated with buffer.

the parent chain. On the other hand, chymotrypsin interacted with α_2-macroglobulin to yield three derivative chains after reduction indicating that there are at least two chymotrypsin susceptible regions in the precursor chain.

α_2-macroglobulin functionally capable of binding enzymes appeared to be required for limiting hydrolytic attack to a specific region of the molecule. The concentration dependent increase in derivative chain demonstrated with trypsin occurred during the 1st min of incubation. No increase in this product was observed during an additional hour of incubation indicating that the enzyme was completely inhibited by α_2-macroglobulin after the initial proteolytic attack on the inhibitor. In contrast, after acid denaturation, α_2-macroglobulin did not bind trypsin and became extremely susceptible to trypsin-induced degradation, being hydrolyzed into multiple low molecular weight products.

Hydrolysis of α_2-macroglobulin accompanied its interaction with proteases but the data do not permit a conclusion as to whether this particular reaction was necessary for the formation of the enzyme-α_2-macroglobulin complex. This alteration in the structure of α_2-macroglobulin appears to be analogous to the structural changes which occur when trypsin interacts with soybean trypsin inhibitor, as well as with a variety of other protein trypsin inhibitors of non-human, nonplasma origin (16). Laskowski and co-workers (36, 37) have demonstrated that the trypsin inhibitor interaction is accompanied by proteolytic cleavage at a single arginine or lysine residue in the inhibitor located within a peptide loop formed by intrachain disulfide bridging. Several different mechanisms have been suggested to explain the nature of the association between trypsin and its protein inhibitors. Laskowski has suggested that a covalent bond is formed between enzyme and inhibitor involving an acyl-enzyme or tetrahedral intermediate in which the active site serine of trypsin forms an ester bond with the carboxyl of the arginine or lysine reactive site of the inhibitor (16). In this reaction the catalytic activity of trypsin is completely inhibited. A contrasting theory has been proposed by Feeney and co-workers who have contended that peptide bond cleavage may be unnecessary for inhibition of trypsin by its inhibitors (38). Regardless of which view proves correct, it is unlikely that a covalent bond is formed between the active site of the enzyme and α_2-macroglobulin since the complex retains enzymic activity.

Nonetheless, peptide bond cleavage may well be a requirement for formation of the α_2-macroglobulin-enzyme complex since a proteolytic step appears to be necessary for this reaction. Whereas plasmin formed a complex with α_2-macro-globulin, neither plasminogen nor DFP-inactivated plasmin were found to do so (39). Furthermore, in unpublished gel sieving experiments, more than 85% of an ^{125}I-labeled trypsin preparation (molecular weight 24,000) was eluted with purified α_2-macroglobulin as a high molecular weight complex, whereas less than 5% of an ^{125}I-labeled DFP-inactivated trypsin preparation eluted with α_2-macroglobulin. Thus, complex formation required the enzymic active site. These investigations parallel prior studies which suggested that the ability of plasmin to alter both the functional and immunoelectrophoretic properties of $\overline{C1}$ inactivator, an inhibitor of plasmin as well as several other plasma enzymes, was due to the proteolytic activity of the enzyme (40).

Jones et al. (41) studied α_2-macroglobulin isolated from plasma by ultra-centrifugal techniques and concluded that the molecular weight of its subunit chain after reduction was 196,000. This molecular weight is in close agreement to the one found in this study by SDS-polyacrylamide gel electrophoresis utilizing α_2-macroglobulin also prepared from plasma. These values stand in sharp contrast to the results of Frénoy et al. (42) who found that 85% of a reduced sample of α_2-macroglobulin isolated from serum had a molecular weight of 83,000; the remaining 15% had a molecular weight of 190,000. The present study provides an explanation for this disparity in the molecular weight esti-

mates of the subunit chains of α_2-macroglobulin (viz., 196,000 vs. 83,000). The lower molecular weight chain observed by Frénoy et al. corresponds to the derivative chain identified in samples of α_2-macroglobulin complexed with proteases in experiments performed with purified components or from plasma activated with urokinase. It is likely that the structure of serum α_2-macroglobulin is a consequence of its interaction with enzymes like thrombin, plasmin, and kallikrein which are activated during the conversion of plasma to serum (43–47).

The data obtained in this study suggest a possible model for the subunit structure of α_2-macroglobulin (Fig. 6). It has been shown that α_2-macroglobulin
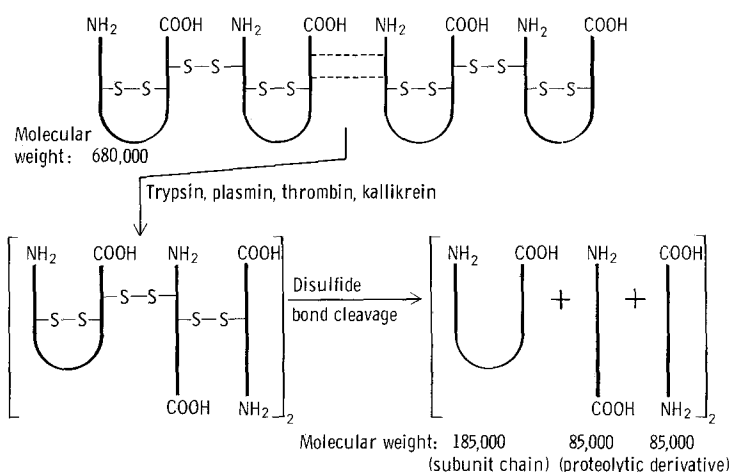

Fig. 6. A diagrammatic model of the proposed subunit structure of human plasma α_2-macroglobulin indicating the modification induced in the molecule by interaction with proteases. The placement of the NH$_2$-terminal and C-terminal ends of the subunit chains is arbitrary and the minimum number of inter- and intrachain disulfide bridges is indicated. The half-molecules of α_2-macroglobulin are held together by noncovalent bonds (dashed lines). In the model shown, one-half of the precursor subunit chains have been cleaved.

dissociated into half-molecules at acid pH or in the presence of urea suggesting that the protein has a noncovalently linked dimeric structure (41, 48–50). Prior studies have estimated the molecular weight of α_2-macroglobulin to be in the order of 650,000 (51) or 725,000 (41). The molecule must necessarily consist of four chains since each subunit chain formed after reduction has a molecular weight of 185,000; each half-molecule, therefore, is comprised of two subunit chains linked by disulfide bridges. The derivative subunit chains resulting from the α_2-macroglobulin-protease interaction were identified only after reduction of the α_2-macroglobulin preparations indicating that they were linked to the parent molecule by both intra- and interchain disulfide bonds. This conclusion is based in part upon the assumption that each of the four subunit chains is

essentially identical. These data indicate that a total of eight such derivative products can be formed per molecule α_2-macroglobulin.

Prior studies have indicated that plasma proteases may be activated and bound to α_2-macroglobulin during the procedures involved in blood collection and protein purification unless appropriate precautions are employed (4, 8). In addition, recent evidence has suggested that a portion of circulating α_2-macroglobulin may be complexed with a plasmin-like proteolytic enzyme (4). Thus, the finding of six different NH_2-terminal amino acids associated with purified α_2-macroglobulin (50) may be due not only to the subunit chain structure of the protein, but also to the bound enzyme(s) as well as to new NH_2-terminal amino acids produced by proteolysis.

This study has demonstrated that α_2-macroglobulin acts as a substrate for circulating proteases and raises the possibility that other plasma inhibitors may interact with the enzymes they inhibit in a similar manner. These findings provide new information regarding the nature of α_2-macroglobulin, a circulating modulator of the coagulation, fibrinolytic, and kallikrein enzyme systems.

SUMMARY

Human plasma α_2-macroglobulin is an inhibitor of circulating proteases that function in hemostatic and inflammatory reactions but the biochemical nature of its interaction with these enzymes is not well defined. This investigation has found that α_2-macroglobulin is comprised of subunit chains of 185,000 molecular weight as analyzed by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Trypsin, thrombin, plasmin, and plasma kallikrein in amounts completely bound to α_2-macroglobulin attacked one region in the subunit chain producing a single derivative with a molecular weight of 85,000 indicating that hydrolysis occurred at or near the center of the parent chain. The proteolytic derivative was also identified in an α_2-macroglobulin preparation from plasma incubated with the plasminogen activator, urokinase. α_2-macroglobulin functionally capable of binding enzyme appeared to be required both for limiting tryptic hydrolysis and for confining the concentration dependent increase in the derivative chain to the 1st min of incubation since acid-denatured α_2-macroglobulin that failed to bind trypsin was extensively degraded. Three derivative chains resulted from the interaction of α_2-macroglobulin with chymotrypsin demonstrating the presence of at least two chymotrypsin susceptible regions in the precursor chain. Reduction of the α_2-macroglobulin-enzyme mixture was required for the identification of the derivative subunit chains establishing that these cleavage products were covalently linked to the parent molecule by disulfide bridges. Thus, α_2-macroglobulin acts as a substrate for circulating proteases, a finding which may also pertain to the mechanism of action of other plasma enzyme inhibitors.

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