Characterization of Functional Human α_2-Macroglobulin Half-Molecules Isolated by Limited Reduction with Dithiothreitol†

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ABSTRACT: Human α_2-macroglobulin was reacted in sequence with 500 μM dithiothreitol (1 h) and 1.3 mM iodoacetamide (40 min) under nondenaturing conditions. The modified protein consisted of a homogeneous population of half-molecules as determined by sedimentation equilibrium and gel filtration chromatography experiments. Amino acid analyses indicated that four disulfide bonds were cleaved and alkylated per subunit. Equivalent reaction conditions caused intersubunit disulfide bond reduction in α_2-macroglobulin–methylamine; however, the protein subunits remained associated as tetramers typical of whole molecules. The α_2-macroglobulin half-molecules bound trypsin reversibly and irreversibly. The modified inhibitor retained 80% of the covalent trypsin binding capacity of an equivalent weight of unmodified tetrameric α_2-macroglobulin. Reaction of α_2-macroglobulin half-molecules with trypsin or methylamine in the presence of iodoacetamide resulted in the generation of one new carboxamidomethylcysteine residue per subunit. This result and the protease covalent incorporation data provide evidence for the integrity of the half-molecule subunit thioester bonds. Electrophoresis experiments demonstrating that amine-treated half-molecules do not fragment at elevated temperature support this conclusion. α_2-Macroglobulin half-molecules reassociated to form tetramers after reaction with trypsin or methylamine. Reassociation may have been favored by the formation of intersubunit disulfide bonds between cysteine residues liberated during thioester cleavage. Trypsin bound to half-molecules reacted more extensively with soybean trypsin inhibitor than trypsin bound to whole molecules (0.32 mol of soybean trypsin inhibitor/mol of trypsin as compared to 0.02 mol/mol). Reaction of half-molecules with methylamine decreased the susceptibility of the subunit bait regions to tryptic cleavage. In vivo plasma elimination experiments demonstrated that the half-molecules retained the potential to generate a functional receptor recognition site after reaction with protease or amine. These studies indicate that α_2-macroglobulin half-molecules may be isolated without disruption of the structure critical to the function of the inhibitor.

α_2-Macroglobulin is a glycoprotein that functions in the binding and inhibition of a wide range of proteases in the circulation of man (Barrett & Starkey, 1973). The native inhibitor is a tetramer of identical subunits (Harpel, 1973; Sottrup-Jensen et al., 1981b; Swenson & Howard, 1979a,b). Disulfide bonds bridge subunits into pairs that have a molecular weight of approximately 360 000 (Harpel, 1973). Strong noncovalent interactions are responsible for the association of pairs into whole molecules of approximately 718 000 daltons (Hall & Roberts, 1978). These noncovalent interactions may be disrupted by high concentrations of denaturant, such as urea or guanidine hydrochloride (Jones et al., 1972; Frenoy et al., 1977).

Conformational change plays a central role in the function of α_2-macroglobulin (Barrett et al., 1979). Reaction with proteases is initiated at the inhibitor "bait region" where a susceptible peptide bond is cleaved by the attacking enzyme (Harpel, 1973; Barrett et al., 1979; Sottrup-Jensen et al., 1981b). α_2-Macroglobulin then undergoes a series of structural changes that physically entrap the protease so that dissociation is prevented (Barrett et al., 1979; Gonias et al., 1982b). A covalent bond between protease and inhibitor may be formed; however, this bond does not involve the protease active site and is not critical for the irreversible association of the two proteins (Salvesen & Barrett, 1980). The site of covalent bond formation on the α_2-macroglobulin subunit is a labile thioester bond (Salvesen et al., 1981; Sottrup-Jensen et al., 1980). Protease lysine residues with low pK ϵ-amino groups are most reactive with the inhibitor thioesters (Salvesen et al., 1981; Wang et al., 1981a).

Small primary amines such as methylamine and ethylamine may react directly with α_2-macroglobulin thioester bonds in the absence of protease (Barrett et al., 1979; Sottrup-Jensen et al., 1980). This reaction also causes a conformational change in the inhibitor (Barrett et al., 1979). The structure of α_2-macroglobulin after reaction with methylamine is very similar to or identical with the structure of the inhibitor bound to protease (Gonias et al., 1982b). Both complexes are bound and endocytosed by a receptor present on macrophages and fibroblasts (Imber & Pizzo, 1981; Kaplan et al., 1981; Van Leuven et al., 1979). This receptor does not recognize unreacted α_2-macroglobulin.

Very little information is available concerning how α_2-macroglobulin subunits interact to form protease binding sites. Each molecule of inhibitor is capable of binding two molecules of trypsin or chymotrypsin irreversibly, suggesting that two distinct binding sites exist (Swenson & Howard, 1979a; Pochon et al., 1981). It is unclear whether two subunits are capable of functioning independently to bind one molecule of protease. It this were true, an intact binding site might result only from the association of either covalently or noncovalently bound subunits.

The disulfide bonds in many proteins may be reduced with dithiothreitol and alkylated with iodoacetamide under nondenaturing conditions without causing extensive denaturation or loss of activity (Liu & Meienhofer, 1968; Bewley et al., 1968; Bewley & Li, 1969). The high redox potential of dithiothreitol permits its use at low concentration compared to other reducing reagents (Cleland, 1964; Bewley & Li, 1969). Carboxamidomethylation with iodoacetamide is preferable to alkylation with iodoacetic acid because the former leaves the distribution of amino acid side chain charges intact (Tanford,

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1968). Jones et al. (1972) reduced α_2-macroglobulin under nondenaturing conditions with reagents other than dithiothreitol. Barrett et al. (1979) reduced α_2-macroglobulin with dithiothreitol and then alkylated with iodoacetic acid. Both groups of investigators obtained quarter molecules (individual subunits) as a final product. This result would seem to imply that the noncovalent forces holding non-disulfide-bonded subunits together are not strong enough to prevent dissociation of isolated half-molecules. Alternatively, the yield of single subunits instead of half-molecules may reflect some degree of protein denaturation. In this report, reduction and alkylation reactions are described that yield functional α_2-macroglobulin half-molecules, each consisting of two noncovalently bound subunits. These half-molecules bind trypsin reversibly, irreversibly, and covalently, demonstrate intact thioester bond chemistry, and undergo structural change after reaction with methylamine or trypsin so that they are recognized by the α_2-macroglobulin receptor. The quarter molecules isolated by Barrett et al. (1979) shared some of the structural characteristics of half-molecules but lacked protease inhibitory activity.

Experimental Procedures

Reagents. Dithiothreitol, *p*-nitrophenyl *p*-guanidinobenzoate, and N^{α}-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) were from Sigma, and phenylmethanesulfonyl fluoride was from Calbiochem. Protein radioiodination was performed with $Na^{125}I$ from New England Nuclear. Ultrogel AcA-22 was obtained from LKB. Pharmacia supplied Sephadex G-150 and G-25. Electrophoresis reagents were purchased from Bio-Rad. All other reagents were of the best commercial grade available.

Proteins. Human α_2-macroglobulin was purified from plasma as described by Kurecki et al. (1979) and modified by Imber & Pizzo (1981). Trypsin was purchased from Worthington Biochemicals. This preparation was 70% active as determined by active-site titration (Chase & Shaw, 1967). Soybean trypsin inhibitor was purchased from Sigma.

Methylaminolysis of α_2-Macroglobulin Thioester Bonds. α_2-Macroglobulin was reacted with 200 mM methylamine for 2 h at pH 8.0 unless otherwise described. The solution was then dialyzed overnight to remove unreacted amine.

Reduction and Alkylation. A 10 mM dithiothreitol solution and a 25 mM iodoacetamide solution were freshly prepared before each experiment. α_2-Macroglobulin (1.0–1.5 μM) was reacted with 500 μM dithiothreitol at 23 °C for 1 h in 120 mM sodium phosphate, pH 7.4. Iodoacetamide was then added to the reaction mixture at a final concentration of 1.3 mM for an additional 40 min. Reduced and alkylated preparations (0.2–1.5 mL) were dialyzed against 4 L of 20 mM sodium phosphate and 100 mM NaCl, pH 7.4. The dialysis buffer was replaced twice at 6-h intervals.

Analysis of Protein Alkylation. The extent of protein carboxamidomethylation achieved under the experimental conditions was determined by amino acid analysis. Alkylated α_2-macroglobulin was hydrolyzed, in vacuo, for 24 h at 110 °C in 6 N HCl. Amino acid compositions were determined in a Beckman 120 B analyzer. For each analysis, measured levels of unmodified amino acids were compared to the published α_2-macroglobulin amino acid analysis (Hall & Roberts, 1978) in order to accurately determine the concentration of hydrolyzed protein. In a control experiment, native unreduced α_2-macroglobulin (1.5 μM) was reacted with 2 mM iodoacetamide for 2 h. No carboxymethylcysteine was detected, confirming the report of other investigators who also found no free thiol groups in unreacted α_2-macroglobulin (Salvesen et al., 1981; Sottrup-Jensen et al., 1980).

Protein Radioiodination. α_2-Macroglobulin, trypsin, and soybean trypsin inhibitor were radioiodinated by the solid-state lactoperoxidase method described by David & Reisfeld (1974). Unreacted iodine was separated from protein by chromatography on Sephadex G-25. Radioactivity was measured in a Scientific Products AW14-120 γ counter with an ^{125}I counting efficiency of 75%. The specific activities of trypsin and soybean trypsin inhibitor were 0.36 and 0.12 μCi/μg, respectively. α_2-Macroglobulin was prepared at both low and high specific activity for chromatography experiments (0.002 and 0.25 μCi/μg) and at a specific activity of 0.56 μCi/μg for in vivo plasma elimination studies.

Polyacrylamide Gel Electrophoresis. Electrophoresis of denatured proteins was conducted in an ammediol-buffered gel system with sodium dodecyl sulfate ($NaDodSO_4$) in the upper reservoir as described by Wyckoff et al. (1977). Phenylmethanesulfonyl fluoride (2 mM) was reacted with samples containing trypsin for 30 min prior to exposure to denaturing conditions. Solutions were incubated for 40 min at 37 °C in $NaDodSO_4$, with or without 10 mg/mL dithiothreitol, and then applied on gels (5% polyacrylamide slabs). Densitometry scans of gels stained with Coomassie Brilliant Blue R-250 were obtained as previously described (Gonias & Pizzo, 1981a).

Gel Filtration Chromatography. Ultrogel AcA-22 and Sephadex G-150 were suspended in columns that were 25 × 1.2 cm and 25 × 0.8 cm, respectively. A constant flow rate of between 1.8 and 3.0 mL/h was maintained for both columns with a peristaltic pump. The chromatography buffer was 20 mM sodium phosphate and 100 mM NaCl, pH 7.4. The elution fraction volume was 400 μL for the Ultrogel AcA-22 column and 300 μL for the Sephadex G-150 column. Greater than 95% of the chromatographed protein was recovered for all reported experiments.

Protein Concentrations. An absorption coefficient ($A^{1\%,1cm}_{280nm}$) of 8.93 was used to determine the concentration of α_2-macroglobulin (Hall & Roberts, 1978). The absorption at λ = 280 nm of α_2-macroglobulin–methylamine is unchanged compared to that of an equivalent concentration of the unreacted inhibitor (Gonias et al., 1982b). The $A^{1\%,1cm}_{280nm}$ of soybean trypsin inhibitor is 10.13 (Yamamoto & Ikenkar, 1967).

The following experiment was performed in triplicate to determine the absorption coefficient for a homogeneous solution of α_2-macroglobulin half-molecules. α_2-Macroglobulin was radioiodinated at low specific activity (5×10^{-4} μCi/μg) and then reduced and alkylated as described above to generate half-molecules. The concentration of protein was determined on the basis of radioactivity measurements. Absorption was measured in a Cary 17 double-beam spectrophotometer. The results of the three equivalent experiments were averaged to yield $A^{1\%,1cm}_{280nm}$ = 9.38.

Amidase Activity Assay. Trypsin that is bound to α_2-macroglobulin retains amidase activity in the presence of soybean trypsin inhibitor. This activity was measured by a modification of the procedure described by Ganrot (1966). Trypsin was reacted with α_2-macroglobulin or modified α_2-macroglobulin at an active protease concentration 2-fold higher than the concentration of potential protease binding sites. The reaction proceeded for 10 min at 23 °C in solution buffered to pH 7.4. Soybean trypsin inhibitor was then added to a final concentration twice that of the total trypsin present in solution for an additional 10 min. Preparations were reacted with the substrate BAPNA at 37 °C for 1 h at pH 8.2. Activity was expressed as the difference in absorbance at λ = 405 nm between the sample and a blank that contained no α_2-

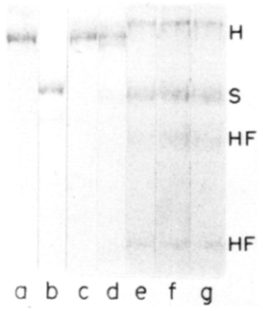

FIGURE 1: Electrophoresis of native α_2-macroglobulin after reduction with dithiothreitol and alkylation with iodoacetamide. The dithiothreitol incubation concentrations were (a) 0, (b) 0, (c) 30 μM, (d) 125 μM, (e) 500 μM, (f) 2 mM, and (g) 20 mM. Iodoacetamide was added to each solution at a final concentration of 0.35 mM higher than 2 times the molarity of the reducing reagent. Reaction conditions and time periods of incubations were as described under Experimental Procedures. All samples were denatured in $NaDodSO_4$, without reductant at 37 °C prior to electrophoresis, except for the protein in lane b which was simultaneously reduced and denatured. Electrophoretic bands referred to in the text are labeled as follows: H, half-molecules; S, single subunits; HF, heat fragmentation bands.

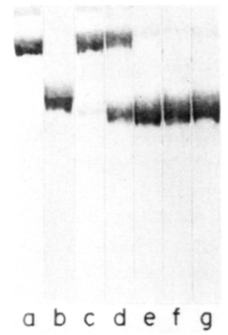

FIGURE 2: Electrophoresis of α_2-macroglobulin–methylamine after reduction and alkylation. The dithiothreitol incubation concentrations were (a) 0, (b) 0, (c) 30 μM, (d) 125 μM, (e) 500 μM, (f) 2 mM, and (g) 20 mM. Reduction and alkylation were performed as described in Figure 1. All samples were denatured without reductant prior to electrophoresis, except for the protein in lane b which was simultaneously reduced and denatured.

macroglobulin but was otherwise identical.

Molecular Weight Determinations. Sedimentation equilibrium experiments were performed in a Spinco Model E ultracentrifuge equipped with a photoelectric scanner. Centrifuge speeds were selected to give a slope of ln A_{280}/r^2 of approximately 1.0–1.5. The density (ρ) of the solvent (20 mM sodium phosphate and 100 mM NaCl, pH 7.4) was 1.0057. The partial specific volume ($\bar{v}$) of α_2-macroglobulin is 0.731 (Hall & Roberts, 1978). At least 48 h was required for centrifuged solutions to reach equilibrium. Final scans demonstrated essentially quantitative recovery of protein in all the reported experiments.

In Vivo Plasma Elimination Studies. Radioiodinated α_2-macroglobulin and modified α_2-macroglobulin preparations were injected into the venous circulation of CD-1 female mice. Equivalent volumes of blood were removed at multiple time points and measured for radioactivity in a γ counter. The radioactivity remaining in the circulation was calculated as a percentage relative to an initial blood sample drawn approximately 10 s after injection. Reported studies were performed in triplicate. Each individual study varied by less than 5% compared to the mean. Detailed descriptions of this procedure may be found elsewhere (Imber & Pizzo, 1981; Gonias et al., 1982a).

Results

Reaction of Dithiothreitol with α_2-Macroglobulin and α_2-Macroglobulin–Methylamine. Reduction of α_2-macroglobulin intersubunit disulfide bonds, under nondenaturing conditions, was first studied as a function of the dithiothreitol concentration. Reduced and alkylated preparations were dialyzed extensively and compared in denaturing gel electrophoresis experiments (Figure 1). Control lanes show the mobilities of half-molecules with two disulfide-bonded subunits (obtained by denaturing α_2-macroglobulin in the absence of reductant) and single subunits (simultaneous reduction and denaturation). Two higher mobility heat fragmentation bands were observed when α_2-macroglobulin was exposed to temperatures above 37 °C under denaturing conditions (Harpel et al., 1979). The internal reaction that is responsible for fragmentation involves cleavage of both a thioester bond and an adjacent peptide bond (Howard et al., 1980; Sottrup-Jensen et al., 1980). Electrophoresis of α_2-macroglobulin samples that were reacted for 1 h with at least 500 μM dithiothreitol showed prominent single subunit bands even though no reductant was present during denaturation. This result indicates that α_2-macroglobulin intersubunit disulfide bonds can be cleaved under nondenaturing conditions. Intersubunit disulfide bond reduction was significantly less complete after incubation with 125 μM dithiothreitol. No single subunits were evident for the reaction with 30 μM reductant. Heat fragmentation bands were present in gel lanes that showed single subunit bands. Evidence is presented below to confirm the expected result that these bands were generated only during the incubation period with $NaDodSO_4$ at 37 °C. Prior reaction with dithiothreitol under nondenaturing conditions may make α_2-macroglobulin more susceptible to temperature-sensitive fragmentation in $NaDodSO_4$.

An equivalent series of reduction and alkylation experiments was performed with α_2-macroglobulin–methylamine. Electrophoresis of denatured samples provided evidence for reduction of intersubunit disulfide bonds at every dithiothreitol concentration tested (30 μM–20 mM) (Figure 2). Reaction with 500 μM dithiothreitol in nondenaturing buffer yielded single subunits in the denaturing gel with nearly the same efficacy as simultaneous denaturation and reduction. These data suggest that the subunit bridging disulfide bonds in α_2-macroglobulin–methylamine may be slightly more reactive with dithiothreitol than the same bonds in the unreacted protease inhibitor. Temperature-sensitive fragmentation was not observed for α_2-macroglobulin–methylamine, in agreement with a previous study (Harpel et al., 1979).

Physicochemical Characterization of α_2-Macroglobulin Half-Molecules. α_2-Macroglobulin was reduced and alkylated as described under *Experimental Procedures*. The modified protease inhibitor was chromatographed on Ultrogel AcA-22, yielding a single symmetric absorption peak. Electrophoresis of multiple elution fractions under denaturing conditions revealed no evidence of heterogeneity. An equivalent preparation of reduced, carboxamidomethylated ^{125}I-labeled α_2-macroglobulin was chromatographed on the same column. A single symmetric radioactivity peak was detected. These studies demonstrate that the products of reduction and alkylation of α_2-macroglobulin, under the conditions described, are homogeneous within the limits of sensitivity of the chromatography procedure.

Sedimentation equilibrium experiments were performed to determine the molecular weight of the modified α_2-macroglobulin. At equilibrium, the graph of ln c vs. r^2 was linear. The molecular weight of the reduced, alkylated α_2-macroglobulin was 352 000. This value is within 2% of the expected

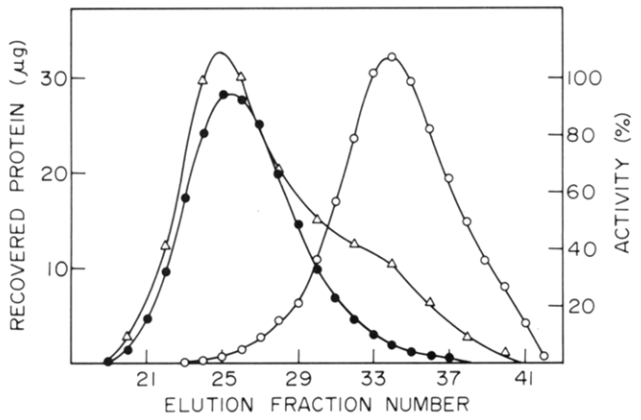

FIGURE 3: Gel filtration cochromatography of α_2-macroglobulin half-molecules and whole molecules. Radioiodinated half-molecules (240 μg) and nonradioactive whole molecules (200 μg) were cochromatographed on Ultrogel AcA-22. Recovery of half-molecules in elution fractions was monitored with radioactivity measurements (○). Total protein was determined by the absorbance at λ = 280 nm. Recovery of whole molecules in elution fractions was calculated as the difference between the two determined values (●). Every second elution fraction was measured for soybean trypsin inhibitor resistant amidolytic activity as described under Experimental Procedures. The fraction containing the greatest activity was arbitrarily referenced as 100% active. Activity in other fractions was expressed as a percentage of the reference fraction (△). In two separate control experiments, 250 μg of half-molecules or whole molecules was chromatographed with 0.5 μg of radioiodinated (high specific activity) whole molecules or half-molecules, respectively. The elution profiles were consistent with that shown in the figure.

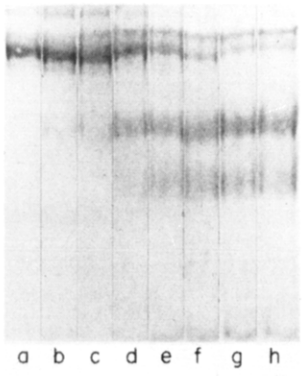

FIGURE 4: Polyacrylamide gel electrophoresis of fractions eluting from the column during the chromatography experiment shown in Figure 3. Protein solutions were denatured in $NaDodSO_4$ without reductant prior to electrophoresis. Each electrophoresis lane contained 6 μg of protein. The fraction numbers correspond directly to the numbers of the *x* axis of Figure 3: (a) fraction 23, (b) 25, (c) 27, (d) 29, (e) 31, (f) 33, (g) 35, and (h) 37.

molecular weight on an α_2-macroglobulin half-molecule (359 000).

The extent of disulfide bond reduction and alkylation obtained during the preparation of half-molecules was assessed with amino acid analysis. For each mole of tetrameric α_2-macroglobulin subjected to the standard reaction conditions, 31.9 mol of carboxymethylcysteine was detected (8.0 mol of carboxymethylcysteine per mol of subunit). This value corresponds to the reduction of 16 disulfide bonds per tetramer. The α_2-macroglobulin half-cystine content determined by Hall & Roberts (1978) was 113 residues/tetramer. It is, therefore, likely that the majority of the α_2-macroglobulin disulfide bonds remained unreduced when half-molecules were prepared, despite the cleavage of the intersubunit covalent linkages. Barrett et al. (1979) detected three to four new thiol groups per subunit with 5,5′-dithiobis(2-nitrobenzoic acid) after reacting α_2-macroglobulin with 1 mM dithiothreitol for 30 min. The investigators determined, however, that the assayed preparation contained an unspecified fraction of intact tetramers.

α_2-Macroglobulin half-molecules and whole molecules were cochromatographed on Ultrogel AcA-22 (Figure 3). Half-molecules of α_2-macroglobulin were clearly resolved from the native tetramers. Fractions from both elution peaks were subjected to denaturing gel electrophoresis without further reduction (Figure 4). The majority of the protein in the half-molecule peak migrated at a rate typical of single subunits, as expected. Some of the half-molecule subunits underwent temperature-sensitive fragmentation when exposed to denaturing conditions at 37 °C. No single subunits were observed when protein from the lead edge of the first chromatography elution peak was electrophoresed. This observation confirms the results of other experiments presented above suggesting that none of the reduced and alkylated α_2-macroglobulin subunits remain associated as tetramers.

Electrophoresis of protein from the half-molecule peak demonstrated an additional band with slightly less mobility than unreduced denatured α_2-macroglobulin (Figure 4). An equivalent band of limited and variable intensity was observed when isolated homogeneous half-molecule preparations were compared by electrophoresis (between 2 and 10% of total protein by densitometry). This suggests that cystine residues in some inhibitor molecules may be reduced but not alkylated so that thiol groups remain available to re-form disulfide bonds during either dialysis or denaturation for electrophoresis. Storage of half-molecules for up to 2 weeks at either 4 or 23 °C did not result in an increase in the protein content of this band. The difference in mobility between unreduced α_2-macroglobulin and half-molecules with re-formed disulfide bonds suggests different extents of denaturation, consistent with previous studies of protein denaturation in $NaDodSO_4$ (Tanford, 1968).

Physicochemical Characterization of Reduced and Alkylated α_2-Macroglobulin–Methylamine. α_2-Macroglobulin–methylamine was reacted with dithiothreitol and iodoacetamide as described above. Chromatography on Ultrogel AcA-22 yielded a single symmetric peak. No heterogeneity was detected when protein from the ascending and descending sides of the peak was subjected to electrophoresis in $NaDodSO_4$. These results paralleled those obtained for native α_2-macroglobulin after reduction and alkylation. Sedimentation equilibrium experiments with reduced, carboxamidomethylated α_2-macroglobulin–methylamine yielded a linear plot of ln *c* vs. r^2 and a molecular weight of 720 000. This is essentially the molecular weight of untreated α_2-macroglobulin (Hall & Roberts, 1978).

Reduced and alkylated α_2-macroglobulin–methylamine was cochromatographed with untreated α_2-macroglobulin–methylamine on Ultrogel AcA-22. The elution positions of the modified and unmodified proteins were identical, confirming the results of the sedimentation equilibrium experiments. Noncovalent interactions are apparently strong enough to maintain α_2-macroglobulin–methylamine in the tetrameric state in the absence of subunit-bridging disulfide bonds. An equivalent observation was made when α_2-macroglobulin–methylamine was reduced and then alkylated with iodoacetic acid despite the introduction of negative charges into the molecule (Barrett et al., 1979).

Reaction of α_2-Macroglobulin Half-Molecules with Trypsin. The fractions recovered from the cochromatography of tetrameric α_2-macroglobulin and α_2-macroglobulin half-molecules, shown in Figure 3, were assayed with the method

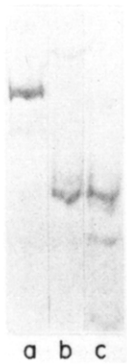

FIGURE 5: Electrophoresis of tetrameric α_2-macroglobulin and half-molecules after reaction with trypsin. (a) Tetrameric α_2-macroglobulin; (b) tetrameric α_2-macroglobulin reacted with trypsin; (c) α_2-macroglobulin half-molecules reacted with trypsin. The three samples were denatured in the presence of reductant.

described by Ganrot (1966). The assay results are superimposed on the elution profile. Significant activity was associated with the first peak containing whole molecules. A low level of activity was also detected for the half-molecules in the second peak. Isolated preparations of half-molecules consistently retained between 15 and 25% of the activity associated with an equivalent weight of whole molecules. The activity of half-molecules may be decreased by any of three mechanisms: (1) fewer molecules of trypsin bind to the modified population of α_2-macroglobulin; (2) bound trypsin is not sterically protected from reaction with soybean trypsin inhibitor; (3) the kinetic parameters for the reaction of BAPNA with trypsin in complex with half-molecules and whole molecules of α_2-macroglobulin are different. The relative contributions of the three mechanisms were studied with radioiodinated trypsin incorporation and gel electrophoresis.

α_2-Macroglobulin half-molecules were reacted for 10 min with a 3-fold molar excess of active trypsin. A control aliquot of intact tetramers was treated equivalently. The products of both reactions were compared in a denaturing gel (Figure 5). Essentially all of the subunits in tetrameric α_2-macroglobulin were cleaved at the "bait region", yielding a major band of M_r ~90 000. A higher molecular weight band reflected the occasional formation of covalent bonds between trypsin and more than one α_2-macroglobulin thioester bond (Sottrup-Jensen et al., 1981c; Salvesen et al., 1981). The subunits in α_2-macroglobulin half-molecules were cleaved by trypsin to yield an equivalent M_r ~90 000 band. A small percentage of the fragments underwent a second proteolysis to yield two new bands.

α_2-Macroglobulin half-molecules were then reacted for 10 min with a 3-fold molar excess of active radiolabeled trypsin. The solution was chromatographed on Sephadex G-150. Radioactivity eluted in two peaks, the first consisting of trypsin bound to inhibitor and the second, free protease. The binding of trypsin to half-molecules was 0.67 mol/mol. The experiment was repeated after incubations of half-molecules with trypsin at 1.5- and 6-fold molar excess. The measured binding was within 5% of the above value in both experiments.

α_2-Macroglobulin half-molecules were reacted in sequence, for 10 min each, with radioiodinated trypsin and soybean trypsin inhibitor at twice the protease concentration. Trypsin binding to the half-molecules was 0.50 mol/mol as measured with Sephadex G-150 chromatography. The retention of complexed trypsin by α_2-macroglobulin half-molecules in the presence of an excess of soybean trypsin inhibitor is evidence for significant high-affinity binding.

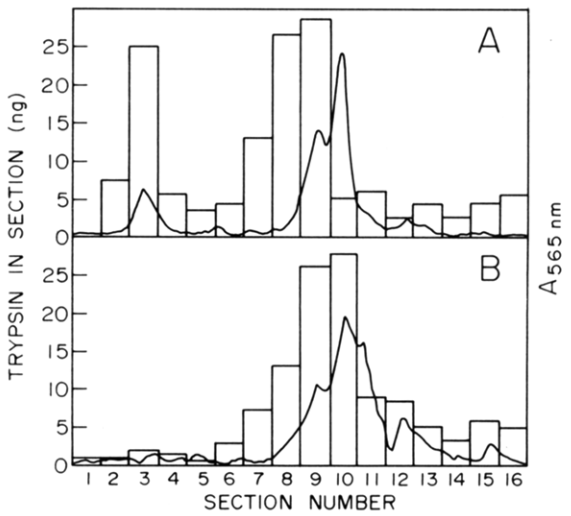

FIGURE 6: Covalent bond formation during the reaction of radioiodinated trypsin with α_2-macroglobulin half-molecules and whole molecules. (A) Tetrameric α_2-macroglobulin was reacted with a 3-fold molar excess of trypsin for 10 min. The trypsin was inactivated, and the sample was denatured in the presence of 10 mg/mL dithiothreitol and subjected to electrophoresis. The gel was stained with Coomassie Brilliant Blue R-250 and scanned with a densitometer at λ = 565 nm. The densitometry tracing is shown with a solid line. The gel lane was sectioned as described in the text. The quantity of trypsin contained in the sections was determined with radioactivity measurements in a γ counter and shown as a bar graph. Sections 17 and 18, which are not shown, contained free trypsin and therefore a large amount of radioactivity. (B) α_2-Macroglobulin half-molecules were reacted with trypsin as described in panel A. The same experiment was then performed. An equivalent quantity of inhibitor (4 μg) was applied to the gels in panels A and B.

α-Macroglobulin half-molecules and tetramers were reacted with radioiodinated trypsin and electrophoresed under denaturing conditions. The stained gels were scanned with a densitometer and then sliced into 18 equivalent sections that were measured for radioactivity in a γ counter (Figure 6). The extent of covalent bond formation between trypsin and the protease inhibitor was determined from the radioactivity recovered at positions in the gel of lower mobility than the free protease, as previously described (Salvesen & Barrett, 1980; Salvesen et al., 1981). The tetramer–trypsin gel contained radioactivity in two low-mobility peaks, corresponding to the position of trypsin covalently bound to one α_2-macroglobulin subunit cleavage fragment (sections 8 and 9) and the position of trypsin covalently bound to two separate subunit cleavage fragments (section 3) (Barrett et al., 1979; Salvesen & Barrett, 1980). The number of moles of trypsin covalently bound to each mole of tetrameric α_2-macroglobulin was 1.06. This result indicates that approximately 50% of the trypsin determined in a previous study to be irreversibly bound to tetrameric α_2-macroglobulin (Gonias et al., 1982b) is covalently bound as well.

The half-molecule–trypsin gel contained a single low-mobility radioactivity peak, corresponding to the position of trypsin covalently bound to one α_2-macroglobulin subunit fragment (M_r ~90 000–110 000). A second peak centered around section 3 was not observed, indicating that the subunit pairs of half-molecules are not cross-linked by trypsin. The number of moles of trypsin covalently bound to each mole of α_2-macroglobulin half-molecules was 0.42. This value represents greater than 80% of the high-affinity trypsin–half-molecule association detected in the presence of soybean trypsin inhibitor with chromatography experiments. Covalent incorporation of proteases by α_2-macroglobulin half-molecules is evidence that the modified inhibitor retains intact thioester

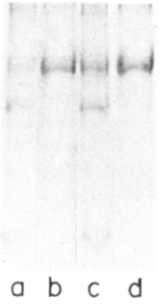

FIGURE 7: Heat-induced fragmentation of α_2-macroglobulin half-molecules and whole molecules. (a) Tetrameric α_2-macroglobulin; (b) tetrameric α_2-macroglobulin–methylamine; (c) α_2-macroglobulin half-molecules; (d) half-molecules after reaction with methylamine. The samples were incubated at 90 °C in nondenaturing solution, cooled, denatured in $NaDodSO_4$ containing reductant, and applied on the gel.

bonds and functional protease binding sites.

Thioester Bond Chemistry. [^{14}C]Methylamine incorporation studies indicate that each subunit in native tetrameric α_2-macroglobulin is capable of binding one molecule of amine (Swenson & Howard, 1979b; Gonias & Pizzo, 1981a). Reactions with proteases and methylamine result in the cleavage of all four subunit thioester bonds in tetrameric α_2-macroglobulin, yielding four new thiol groups that may be alkylated with iodoacetic acid (Sottrup-Jensen et al., 1980; Salvesen et al., 1981). Amino acid analysis experiments were performed in order to confirm the integrity of the half-molecule thioester bonds. α_2-Macroglobulin half-molecules (2.0 μM) were reacted with 2 mM iodoacetamide for 2 h at 23 °C. The number of carboxymethylcysteine residues detected remained unchanged from that reported above. An equivalent preparation of half-molecules was reacted simultaneously with 200 mM methylamine and 2 mM iodoacetamide for 2 h. These reaction conditions increased the carboxymethylcysteine content from 16.0 to 18.1 residues per half-molecule (one new residue per subunit). An equivalent result was obtained when the half-molecules were reacted with a 2-fold molar excess of trypsin in the presence of alkylating reagent (18.0 residues of carboxymethylcysteine per half-molecule; one new residue per subunit). These results provide further evidence that the thioester bonds in α_2-macroglobulin half-molecules are preserved in both structure and function.

Cleavage of tetrameric α_2-macroglobulin subunit thioester bonds by methylamine precludes temperature-sensitive fragmentation, which can otherwise be induced by incubating the protease inhibitor at 90 °C or above in nondenaturing solution or at 37 °C or above in $NaDodSO_4$ (Harpel et al., 1979). Experiments were performed to extend these observations to half-molecules (Figure 7). α_2-Macroglobulin half-molecules were reacted with methylamine and dialyzed extensively. The amine-treated half-molecules, unreacted half-molecules, native tetramers, and tetrameric α_2-macroglobulin–methylamine were heated at 90 °C for 20 min. Electrophoresis of the unreacted half-molecules and whole molecules showed prominent heat fragmentation bands. Half-molecules that were reacted with methylamine did not fragment, analogous to the amine-treated tetramers. These data provide additional support for the conclusion that thioester bonds function equivalently in α_2-macroglobulin half-molecules and whole molecules. The absence of heat fragmentation bands on electrophoresis of the half-molecules reacted with methylamine indicates that the fragmentation observed for unreacted half-molecules did not occur prior to denaturation at 37 °C.

Reassociation of α_2-Macroglobulin Half-Molecules.

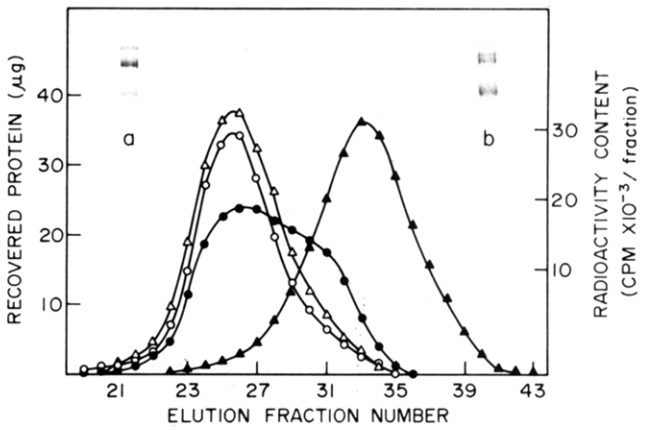

FIGURE 8: Gel filtration chromatography experiments on Ultrogel AcA-22 comparing the elution positions of α_2-macroglobulin half-molecules before and after reaction with methylamine. The results of two experiments are shown. In the first experiment, 275 μg of unreacted nonradioactive half-molecules was cochromatographed with 0.5 μg of radioiodinated tetrameric α_2-macroglobulin–methylamine. Recovery of half-molecules in elution fractions (in micrograms) (▲) was determined by the absorbance at λ = 280 nm. The α_2-macroglobulin–methylamine was detected with radioactivity measurements (△). In the second experiment, 220 μg of nonradioactive α_2-macroglobulin half-molecules was reacted with 200 mM methylamine for 90 min at pH 8.0. The products were dialyzed against 20 mM sodium phosphate and 100 mM NaCl, pH 7.4, for 3.5 h and cochromatographed on Ultrogel AcA-22 with 260 μg of radioiodinated tetrameric α_2-macroglobulin–methylamine. Recovery of reacted tetramers in elution fractions (in micrograms) (○) was determined with radioactivity measurements. The reacted half-molecule content of the elution fraction (in micrograms) (●) was calculated as the difference between the total absorbance at λ = 280 nm and the absorbance attributed to tetramers. The elution fractions from the second experiment were denatured in $NaDodSO_4$ without reductant and subjected to electrophoresis. Gel a contains 5 μg of protein from fraction 24, and gel b contains 5 μg of protein from fraction 30. A third experiment duplicated the procedure of the second, except that 0.5 μg of high specific activity tetrameric α_2-macroglobulin–methylamine was substituted for the low specific activity radioiodinated tetrameric inhibitor. The resulting elution profile was equivalent to that shown in the figure.

Barrett et al. (1979) reported that isolated single subunits reassociate after incubation with trypsin or methylamine. Chromatography experiments were performed to determine whether half-molecules behave similarly under equivalent conditions. Unreacted half-molecules and tetrameric α_2-macroglobulin–methylamine were cochromatographed on Ultrogel AcA-22 (Figure 8). The Stokes radius of the tetrameric α_2-macroglobulin–methylamine complex is 10% smaller than the radius of the unreacted whole molecule (Gonias et al., 1982b). The difference in effective size between unreacted half-molecules and tetrameric α_2-macroglobulin–methylamine was still large enough for adequate resolution.

α_2-Macroglobulin half-molecules were then reacted with methylamine and cochromatographed with tetrameric α_2-macroglobulin–methylamine. The half-molecules that were reacted with amine eluted more rapidly than unreacted half-molecules, overlapping tetrameric α_2-macroglobulin–methylamine (Figure 8). This large increase in the Stokes radius is strong evidence for the reassociation of half-molecules to form whole molecules. Fractions from this chromatography experiment were subjected to electrophoresis (shown in insets). The late eluting protein included a prominent single subunit band. A smaller percentage of the early eluting protein migrated in this position, suggesting that half-molecule reassociation, although extensive, was incomplete or that the reassociated molecules were in rapid equilibrium with nonreassociated forms.

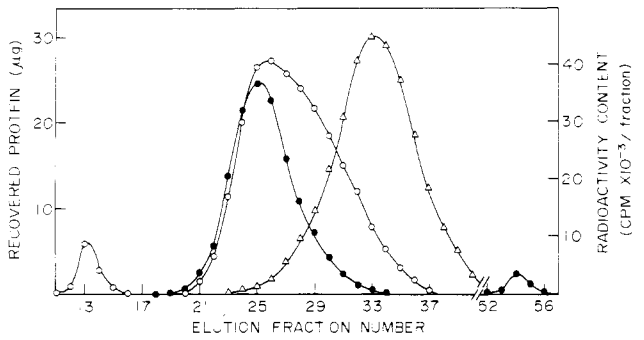

FIGURE 9: Gel filtration chromatography of α_2-macroglobulin half-molecules before and after reaction with trypsin on Ultrogel AcA-22. Radioiodinated tetrameric α_2-macroglobulin was reacted with a 3-fold molar excess of active trypsin for 10 min. Soybean trypsin inhibitor at twice the trypsin concentration was added to the solution for an additional 10 min. The α_2-macroglobulin–trypsin complex was resolved from unreacted trypsin and trypsin bound to the second inhibitor by gel filtration on Sephadex G-150. Less than 1 μg of the purified complex was cochromatographed with 215 μg of unreacted nonradioactive half-molecules in a first experiment. Recovery of half-molecules in elution fractions (in micrograms) (Δ) was determined by the absorbance at λ = 280 nm. The elution position of the tetramer–trypsin complex was determined by measuring fractions for radioactivity (●). In the second experiment, 245 μg of half-molecules was reacted with trypsin for 2 h and then chromatographed (○). This column was calibrated by including an equivalent aliquot of the radioiodinated tetrameric inhibitor–trypsin complex in the applied solution. The radioactivity peak detected near the expected elution position of salt may contain the small glycopeptide Pan et al. (1980) described as released from α_2-macroglobulin during reaction with trypsin. A comparable peak was not observed when radioiodinated tetrameric α_2-macroglobulin was chromatographed before reaction with protease.

Chromatography experiments were performed to study the association properties of α_2-macroglobulin half-molecules after reaction with trypsin (Figure 9). Unreacted half-molecules resolved from tetrameric α_2-macroglobulin–trypsin. This is an expected result since the Stokes radius of tetrameric α_2-macroglobulin–trypsin is only slightly smaller than the radius of the unreacted inhibitor and considerably larger than the radius of α_2-macroglobulin–methylamine (Gonias et al., 1982b). Nearly 95% of the half-molecules that were reacted with trypsin for 2 h eluted in a position overlapping the tetramer–trypsin complex, providing strong evidence for reassociation. The presence of tailing in the half-molecule peak indicates that the reassociation reaction was incomplete, as was the case after reaction with methylamine. Longer periods of incubation with protease prior to chromatography did not cause a further shift in the elution position of the half-molecules, indicating that the reassociation reaction was probably near equilibrium at 2 h. A small fraction of the half-molecules that were reacted with trypsin (less than 5%) chromatographed at a high molecular weight position, suggesting formation of some high molecular weight aggregates.

Incorporation of Soybean Trypsin Inhibitor into Complexes between Half-Molecules and Trypsin. The reaction of soybean trypsin inhibitor with free trypsin is extremely rapid ($k = 1.2 \times 10^7$ M^{-1} s^{-1}) (Bieth et al., 1981). By contrast, trypsin that is irreversibly trapped within tetrameric α_2-macroglobulin reacts extremely slowly with the second inhibitor (Bieth et al., 1981; Wang et al., 1981b). Complex formation between trypsin bound to half-molecules or whole molecules of α_2-macroglobulin and soybean trypsin inhibitor was compared in the following experiments. Tetrameric α_2-macroglobulin was reacted sequentially, for 10 min each, with trypsin and radioactively labeled soybean trypsin inhibitor prior to gel filtration. Radioactivity eluted in three peaks, the first corresponding to soybean trypsin inhibitor bound to α_2-macroglobulin–trypsin followed by two overlapping peaks corresponding to soybean trypsin inhibitor–trypsin and the free inhibitor. α_2-Macroglobulin–trypsin bound 0.04 mol of soybean trypsin inhibitor/mol, as determined from the radioactivity eluting in the first peak. This value indicates that 98% of the protease in complex with tetrameric α_2-macroglobulin retained an unblocked active site.

Table I: Reaction of α_2-Macroglobulin-Bound Trypsin with Soybean Trypsin Inhibitor

form of α_2-macroglobulin	time of reaction: α_2-macroglobulin and trypsin[a]	time of reaction: soybean trypsin inhibitor	soybean trypsin inhibitor bound to α_2-macroglobulin–trypsin: mol/mol of α_2-macroglobulin	soybean trypsin inhibitor bound to α_2-macroglobulin–trypsin: mol/mol of bound trypsin
whole molecules	10 min	10 min	0.04	0.02[b]
half-molecules	2 min	10 min	0.18	0.36[c]
half-molecules	10 min	10 min	0.16	0.32
half-molecules	60 min	10 min	0.13	0.26
half-molecules	2 h	10 min	0.13	0.26

[a] α_2-Macroglobulin was reacted with a 3-fold molar excess of trypsin before addition of radioiodinated soybean trypsin inhibitor at twice the trypsin concentration. The solution was chromatographed on Sephadex G-150. [b] Value calculated on the basis of 2.1 mol of trypsin/mol of α_2-macroglobulin in the tetrameric α_2-macroglobulin–trypsin complex. [c] Value calculated on the basis of 0.5 mol of trypsin/mol of half-molecules in complex incubated with soybean trypsin inhibitor.

α_2-Macroglobulin half-molecules were reacted sequentially with trypsin and soybean trypsin inhibitor for the time intervals indicated in Table I. The longest incubation with trypsin was 2 h, at which time the reassociation reaction had reached equilibrium. Trypsin that was bound to half-molecules reacted more extensively with soybean trypsin inhibitor than protease in complex with tetramers. The difference was 16-fold after incubation of the α_2-macroglobulin half-molecules with trypsin for 10 min. The reactivity of soybean trypsin inhibitor with half-molecule–trypsin complexes decreased as the incubation time prior to addition of the second inhibitor was increased. This result may reflect increased steric hindrance at the protease active site in the reassociated form of α_2-macroglobulin–trypsin. Soybean trypsin inhibitor still bound to 26% of the complexed trypsin when added 2 h after the incubation of trypsin with the half-molecules was initiated.

Role of Subunit Thiol Groups in Half-Molecule Reassociation after Reaction with Amine. α_2-Macroglobulin half-molecules were reacted with methylamine and dialyzed at ambient temperature. Aliquots were removed at various times for electrophoresis. The first lane of Figure 10 shows the preparation of half molecules before reaction with amine. Only a small percentage of the inhibitor subunits were covalently linked by disulfide bonds. This result remained unchanged when the unreacted half-molecules were exposed to pH 8.0 for 6 h. After reaction with methylamine, a significant fraction of the half-molecules migrated with decreased mobility, suggesting interpolypeptide disulfide bond formation between adjacent subunits in the reassociated tetramers. Disulfide-bonded subunit pairs were not observed when half-molecules were reacted with methylamine in the presence of 2 mM iodoacetamide, consistent with the above interpretation.

Susceptibility of α_2-Macroglobulin–Methylamine to Cleavage by Trypsin. Barrett et al. (1979) reported that the bait regions of tetrameric α_2-macroglobulin and α_2-macro-

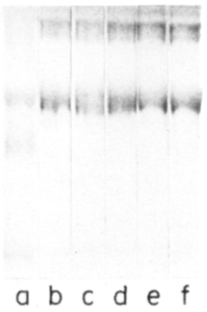

FIGURE 10: Polyacrylamide gel electrophoretic analysis of the reaction of half-molecules with methylamine. Aliquots (5 μg) were removed from a single protein solution at the following time points: (a) before reaction with methylamine; (b) after reaction with 200 mM methylamine for 90 min at pH 8.0. (dialysis was begun immediately after withdrawal of this sample); (c–f) 1, 2, 3, and 4 h, respectively, after dialysis was begun. All samples were denatured in solutions not containing reductant.

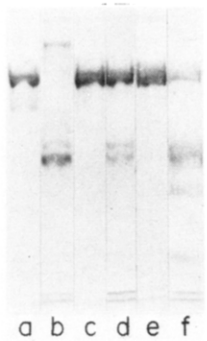

FIGURE 11: Reactivity of trypsin with α_2-macroglobulin–methylamine. All samples were reduced and denatured simultaneously prior to electrophoresis. (a) Native, unreacted, tetrameric α_2-macroglobulin; (b) tetrameric α_2-macroglobulin reacted with trypsin (10 min); (c) tetrameric α_2-macroglobulin–methylamine; (d) tetrameric α_2-macroglobulin–methylamine reacted with trypsin (1 h); (e) α_2-macroglobulin half-molecule–methylamine; (f) α_2-macroglobulin half-molecule–methylamine reacted with trypsin (1 h). The reaction procedures are described in the text.

globulin–methylamine are cleaved by trypsin at the same rate with the formation of equivalent fragments. Wang et al. (1981a) presented data suggesting that tryptic cleavage of α_2-macroglobulin–methylamine is a significantly slower reaction. Figure 11 compares the reactivity of trypsin with the bait regions of tetrameric α_2-macroglobulin–methylamine and inhibitor half-molecules that were reacted with methylamine. Both amine-treated proteins were incompletely cleaved after reaction with trypsin for 1 h. This result differs considerably from that obtained when either native tetramers or unreacted half-molecules were reacted with trypsin. The amine-treated half-molecules were more reactive with trypsin than tetrameric α_2-macroglobulin–methylamine, probably reflecting some differences in structure. The observations in Figure 11 were confirmed at pH 7.4 and pH 8.0, with and without dialysis to remove unreacted methylamine.

In Vivo Probe of the α_2-Macroglobulin Receptor Recognition Site. α_2-Macroglobulin–trypsin and α_2-macroglobulin–methylamine clear rapidly from the circulation of mice compared to the unreacted inhibitor, reflecting binding and endocytosis by a receptor system present on reticuloendothelial cells (Imber & Pizzo, 1981). Figure 12 shows the results of clearance and clearance competition experiments performed to determine whether a receptor recognition site remains intact on α_2-macroglobulin half-molecules. Radioiodinated α_2-macroglobulin was reduced, carboxamidomethylated, and dialyzed extensively. The half-molecules

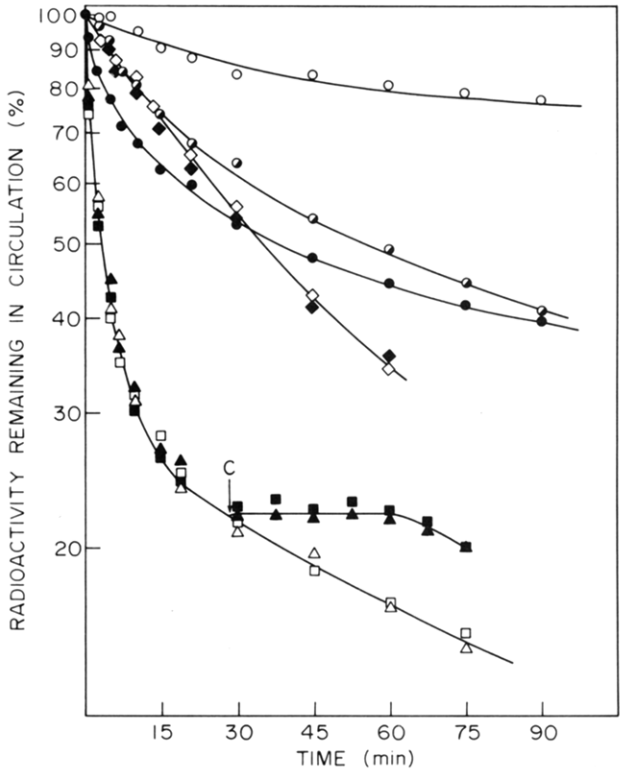

FIGURE 12: In vivo plasma elimination experiments comparing α_2-macroglobulin half-molecules and whole molecules. In each experiment, 1 μg of radioiodinated protein was cleared. Competing ligands were nonradioactive. The plotted studies include the following: tetrameric α_2-macroglobulin (unreacted) (○); unreacted half-molecules (●); unreacted half-molecules in the presence of a 1000-fold molar excess of tetrameric α_2-macroglobulin–trypsin (◑); half-molecules reacted with trypsin (△); half-molecules reacted with methylamine (□); half molecules reacted with trypsin and injected in the presence of a 500-fold molar excess of tetrameric α_2-macroglobulin–trypsin (◇); half-molecules reacted with methylamine and injected in the presence of a 500-fold molar excess of tetrameric α_2-macroglobulin–trypsin (◆); half-molecules reacted with trypsin (▲) and methylamine (■) and cleared without competing ligand for 28 min. At the time point labeled C, a 1000-fold molar excess of tetrameric α_2-macroglobulin–trypsin was administered into the circulation with a second injection. The molar excess of competing ligand was approximated by assuming total reassociation of α_2-macroglobulin half-molecules reacted with trypsin or methylamine.

cleared at a rate intermediate between those of unreacted tetrameric α_2-macroglobulin and α_2-macroglobulin–trypsin. The clearance of the unreacted half-molecules was repeated in the presence of a 1000-fold molar excess of tetrameric α_2-macroglobulin–trypsin complex. The competing ligand would be expected to block radioligand clearance mediated by the α_2-macroglobulin receptor. A low but consistent level of competition was observed, suggesting that unreacted half-molecules may interact weakly with the α_2-macroglobulin receptor. A higher concentration of competing ligand (2000-fold) caused no further competition, indicating that unreacted half-molecules may be cleared by more than one mechanism.

Radioiodinated α_2-macroglobulin half-molecules were reacted with a 2-fold molar excess of trypsin for 10 min, 1 h, and 2 h. The clearances of the three preparations were essentially equivalent to each other and to the clearance of tetrameric α_2-macroglobulin–trypsin complex presented elsewhere (Imber & Pizzo, 1981; Gonias et al., 1982a). Significant competition was observed when the experiment was repeated after injection of a 500-fold molar excess of unlabeled tetrameric α_2-macroglobulin–trypsin. Competition was concentration dependent; lower concentrations of unlabeled ligand

caused less competition (data not shown). Equivalent results are shown in Figure 12 for experiments performed with radioiodinated half-molecules after reaction with methylamine. These experiments show that the potential to generate an intact and completely active receptor recognition site after reaction with protease or amine is maintained by α_2-macroglobulin half-molecules.

Clearance competition experiments may be modified to determine whether plasma elimination of a radioligand is the result of receptor binding and endocytosis or receptor binding alone (Regoeczi et al., 1978; Pizzo & Pasqua, 1982). Asialotransferrin is cleared on receptors that bind but do not internalize the modified protein (Regoeczi et al., 1978; Pizzo & Pasqua, 1982). Injection of a competing concentration of unlabeled asialotransferrin, shortly after the clearance of a dose of the radioiodinated protein is apparently complete, results in the redistribution of the radioligand back into the plasma (Pizzo & Pasqua, 1982). Similar experiments were performed with α_2-macroglobulin half-molecules after reaction with trypsin or methylamine. A competing dose of tetrameric α_2-macroglobulin–trypsin was injected after 75% of either radioiodinated half-molecule preparation had cleared from the circulation. The competing ligand did not promote redistribution of radioactivity back into the plasma, indicating that radioligand endocytosis had occurred.

Discussion

The "trap hypothesis" was initially proposed to explain how α_2-macroglobulin binds proteases irreversibly without necessarily forming a covalent bond (Barrett & Starkey, 1973; Barrett et al., 1979). Trapping refers to a conformational change that occurs in the inhibitor during reaction with protease so that dissociation is prevented by steric hindrance. The conformation of α_2-macroglobulin–methylamine is structurally very similar to the conformation of the inhibitor in complex with trypsin (Gonias et al., 1982b; Barrett et al., 1979). Barrett et al. (1979) reported a procedure that dissociated native α_2-macroglobulin into single subunits. Reacted forms of α_2-macroglobulin remained associated as tetramers even though the same disulfide bonds were apparently reduced and alkylated. A similar procedure for modifying native α_2-macroglobulin is described here that preserves certain subunit interactions so that the resulting products are half-molecules. α_2-Macroglobulin–methylamine resisted dissociation when subjected to this procedure. These results most likely reflect more extensive subunit binding interactions in the reacted as opposed to the unreacted conformation of α_2-macroglobulin. The greater reactivity of subunit cross-linking reagents including glutaraldehyde (Barrett et al., 1979) and *cis*-dichlorodiammineplatinum(II) (Gonias & Pizzo, 1981a,b) with reacted forms of α_2-macroglobulin may also reflect larger areas of close contact.

The number of moles of protease that will react with a mole of α_2-macroglobulin depends on the protease selected; however, many small proteases such as trypsin and chymotrypsin are bound at a ratio of 2:1 (protease:inhibitor) (Pochon et al., 1978; Barrett et al., 1979). Each protease becomes situated in one of two apparently adjacent protease binding sites (Pochon et al., 1981). These observations have led many investigators to postulate that two isolated inhibitor subunits may form an intact protease binding site. One approach for exploring this possibility was to partially denature α_2-macroglobulin in 3 M urea (Barrett et al., 1979). The half-molecules that were obtained after the denaturant was removed with dialysis retained no protease inhibitory activity. Reduction and alkylation of α_2-macroglobulin, as described in this manuscript, yielded half-molecules that bound trypsin both reversibly and irreversibly. It is difficult to accurately determine the extent of reversible protease binding by half-molecules, since chromatography favors dissociation of ligands bound with low affinity. The value presented for total binding of trypsin to half-molecules (0.67 mol/mol) is most likely a minimum estimate. The data presented for covalent incorporation of trypsin into tetramers and half-molecules were less liable to underestimation. α_2-Macroglobulin half-molecules covalently bound 80% of the trypsin covalently bound by an equivalent weight of tetramers. The covalent reaction site on α_2-macroglobulin (the thioester bond) decays rapidly after proteolytic cleavage of the bait region ($t_{1/2}$ = 112 s) as a result of reaction with nucleophiles and hydrolysis by water (Salvesen et al., 1981). Covalent bond formation between protease and inhibitor requires proper orientation of the two proteins so that the inhibitor thioesters preferentially react with protease nucleophilic groups (Salvesen et al., 1981). The essentially unchanged capacity of α_2-macroglobulin half-molecules to covalently bind trypsin indicates that the structural elements of the inhibitor responsible for proper orientation of protease are preserved. It is therefore concluded that half-molecules retain relatively intact and functional protease binding sites.

α_2-Macroglobulin half-molecules retained only one-fourth of the protease binding activity demonstrated by tetramers when assayed by the method of Ganrot (1966). The decrease in measured activity may be attributed largely to reactions of the inhibitor–trypsin complex with soybean trypsin inhibitor. This second inhibitor facilitated the dissociation of a large fraction of the trypsin noncovalently bound to half-molecules and/or reassociated tetramers. The residual trypsin binding activity of half-molecules in the presence of soybean trypsin inhibitor was less than 50% of that demonstrated by whole molecules. A second significant fraction of the trypsin bound to α_2-macroglobulin half-molecules was active site inhibited by the soybean inhibitor. Chromatography experiments detected soybean trypsin inhibitor bound to 32% of the trypsin in complex with half-molecules when the reaction conditions for the Ganrot assay were duplicated. It is conceivable that additional soybean trypsin inhibitor became loosely associated with trypsin active sites, inhibiting substrate hydrolysis but dissociating during chromatography. The remaining loss in assay activity is accounted for, assuming that 18% of the trypsin in complex with half-molecules formed this type of loose ternary complex with soybean trypsin inhibitor.

The covalent incorporation of methylamine into tetrameric α_2-macroglobulin and the resultant conformational change occur at different rates (Van Leuven et al., 1982). The conformational change and loss of protease binding activity during reaction with amine follow equivalent pseudo-first-order kinetics, suggesting that the functional change is a direct consequence of the alteration in conformation (Gonias et al., 1982b). One possible explanation for this relationship is that the protease binding sites are partially buried in α_2-macroglobulin–methylamine. The same steric hindrance that prevents dissociation of trypsin in the α_2-macroglobulin–trypsin complex may prevent protease from irreversibly associating with the inhibitor that has reacted with methylamine. Decreased accessibility of the bait region in tetrameric α_2-macroglobulin–methylamine to tryptic cleavage is consistent with this model. The bait region is an important component of the protease binding site that occupies a different position relative to other regions of the protein in the two conformations of α_2-macroglobulin.

α_2-Macroglobulin half-molecules reassociated extensively, re-forming tetramers, after reaction with trypsin or methylamine. Some reassociated tetramers may have been stabilized by covalent disulfide bonds between thiol groups that were not available for bond formation in the unreacted half-molecules. Cysteine residues that are released from thioester bonds during reaction with protease or amine represent the most likely source of these thiol groups. The formation of intersubunit disulfide bonds between cysteine residues derived from thioesters would indicate that these residues are near the surface of the subunit in contact with an associated subunit as opposed to being buried in the protein core.

Reassociated tetramers are most likely somewhat anomalous compared to unreduced α_2-macroglobulin after reaction with trypsin or methylamine. Evidence for this suggestion was obtained in two separate experiments. Trypsin, when reacted with half-molecules, bound significantly more soybean trypsin inhibitor than tetrameric α_2-macroglobulin–trypsin, even after half-molecule reassociation had reached equilibrium. Complexes of half-molecules with methylamine were less vulnerable to tryptic cleavage than unreacted half-molecules but substantially more vulnerable than tetrameric α_2-macroglobulin–methylamine.

Whether the isolated half-molecules consist of subunits that were covalently or noncovalently associated before reduction cannot be definitively addressed with the present data. It is conceivable that the noncovalent interactions between disulfide-bonded subunits are of sufficient magnitude to prevent dissociation in the absence of the disulfide bonds. Half-molecules could then be formed by the dissociation of subunits that were noncovalently bound in the tetramer if reduction and alkylation indirectly weakened critical binding interactions. This model of half-molecule formation is considered highly improbable. It is much more likely that reduction separates previously covalently bound subunits without disturbing the interactions between the noncovalently associated subunits.

The primary structure of α_2-macroglobulin includes at least three separate regions that are critical to function. The bait region, where proteolytic attack is initiated, and the thioester bond site, where covalent attachment of ligands occurs, have been extensively studied in numerous laboratories. Sequence information is available for these two sites (Sottrup-Jensen et al., 1981a,b). Less information is available concerning the nature of the third region that functions in receptor binding. The plasma elimination studies demonstrated that α_2-macroglobulin half-molecules retain the potential to generate a functional receptor recognition site. Additional data were presented to confirm the integrity of the half-molecule bait regions and thioester bonds. It may therefore be concluded that the procedure for modifying α_2-macroglobulin presented in this paper causes no major perturbations in the structure of regions critical to the function of the inhibitor.

Registry No. Trypsin, 9002-07-7; methylamine, 74-89-5; soybean trypsin inhibitor, 9078-38-0.

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Identification of Specific Carboxylate Groups on Cytochrome *c* Oxidase That Are Involved in Binding Cytochrome *c*†

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ABSTRACT: Modification of beef heart cytochrome *c* oxidase with 1-ethyl-3-[3-[^{14}C](trimethylamino)propyl]carbodiimide ([^{14}C]ETC) was found to dramatically inhibit the high-affinity phase of the reaction with cytochrome *c*. Reaction conditions leading to a 50% inhibition of V_{max} resulted in a 12-fold increase in the K_m for cytochrome *c*. This inhibition was accompanied by the incorporation of 1.2 ± 0.3 mol of [^{14}C]ETC into subunit II and much smaller levels of incorporation into the other subunits. The sites labeled by [^{14}C]ETC were determined by hydrolyzing subunit II with trypsin in the presence of 1% octyl β-D-glucopyranoside and separating the resulting peptides by reverse-phase high-pressure liquid chromatography. The tryptic peptides were then further hydrolyzed with *Staphylococcus aureus* protease to determine that Glu-18, Asp-112, Glu-114, and Glu-198 were the major residues labeled. Binding one molecule of cytochrome *c* to cytochrome *c* oxidase dramatically protected Asp-112, Glu-114, and Glu-198 from labeling by [^{14}C]ETC and prevented the loss in electron-transfer activity. We propose that the negatively charged carboxylates on Asp-112, Glu-114, and Glu-198 are involved in cytochrome *c* binding and that their conversion to bulky, positively charged ETC-carboxyl groups inhibits the reaction with cytochrome *c*. Asp-112 and Glu-114 are located in a highly conserved sequence (104-115) containing alternating acidic and aromatic residues. These aromatic residues could serve as an electron-transfer pathway from cytochrome *c* to cytochrome *a* or the EPR-visible copper. Glu-198 is located between the conserved cysteines-196 and -200 which have been proposed to serve as ligands for the EPR-visible copper.

The electron-transfer reaction between cytochrome *c* and cytochrome *c* oxidase is generally thought to involve the formation of a 1:1 complex stabilized by electrostatic interactions. Extensive chemical modification studies have shown that seven or eight highly conserved lysine residues immediately surrounding the heme crevice of cytochrome *c* are involved in the interaction with cytochrome *c* oxidase (Smith et al., 1977; Ferguson-Miller et al., 1978; Rieder & Bosshard, 1980), cytochrome c_1 (Ahmed et al., 1978), cytochrome *c* peroxidase (Kang et al., 1978; Smith & Millett, 1980), and cytochrome b_5 (Stonehuerner et al., 1979). X-ray crystallographic studies have identified a ring of negatively charged carboxylates surrounding the heme crevice of both cytochrome b_5 and cytochrome *c* peroxidase that is complementary to the ring of positively charged lysine residues on cytochrome *c* (Salemme, 1976; Poulos & Kraut, 1980). Recent cross-linking studies have supported the proposal that the ring of carboxylates on cytochrome *c* peroxidase forms the cytochrome *c* binding site (Bisson & Capaldi, 1981; Waldmeyer et al., 1982).

It is reasonable to expect that the cytochrome *c* binding site on cytochrome *c* oxidase would also involve a complementary ring of carboxylate residues. Recent attempts to localize the cytochrome *c* binding site have not been designed to identify specific carboxylate residues but have instead focused on which subunits of cytochrome *c* oxidase are involved. Arylazidolysine-13 cytochrome *c* was found to specifically cross-link to subunit II and block the high-affinity binding site for cytochrome *c* (Bisson et al., 1980). The major site of insertion of the photoactivated azide was found to be His-161 (Bisson et al., 1982a). In contrast, yeast cytochrome *c* modified at Cys-107 on the backside of the protein was cross-linked to subunit III of cytochrome *c* oxidase from both yeast (Moreland & Dockter, 1981) and beef heart (Fuller et al., 1981). On the basis of the above results, we have proposed (Capaldi et al., 1982) that cytochrome *c* binds at a cleft formed at the interface between two monomers of the cytochrome oxidase dimer (Fuller et al., 1979). The ring of lysines surrounding the heme crevice at the front of cytochrome *c* would interact with subunit II of one monomer, while the backside would be close to subunit III on the other monomer.

In the present study, we have used the reagent 1-ethyl-3-[3-[^{14}C](trimethylamino)propyl]carbodiimide ([^{14}C]ETC)[1] to identify specific carboxylate groups on cytochrome *c* oxidase that are involved in cytochrome *c* binding. We previously found that modification of cytochrome *c* oxidase with [^{14}C]-ETC resulted in inhibition of the high-affinity phase of the reaction with cytochrome *c* and incorporation of ^{14}C into subunit II (Millett et al., 1982). Equimolar concentrations of cytochrome *c* dramatically protected cytochrome *c* oxidase from both the inhibition of electron-transfer activity and the

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[1] Abbreviations: [^{14}C]ETC, 1-ethyl-3-[3-[^{14}C](trimethylamino)-propyl]carbodiimide iodide; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; EPR, electron paramagnetic resonance.

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