Subunits of Human α_2-Macroglobulin Produced by Specific Reduction of Interchain Disulfide Bonds with Thioredoxin†

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ABSTRACT: Disulfide bonds in α_2-macroglobulin (α_2M) were reduced with the thioredoxin system from *Escherichia coli*. Under the conditions selected, 3.5–4.1 disulfide bonds were cleaved in each α_2M molecule, as determined by the consumption of NADPH during the reaction and by the incorporation of iodo[^{3}H]acetate into the reaction product. This extent of disulfide bond reduction, approximately corresponding to that expected from specific cleavage of all four interchain disulfide bonds of the protein, coincided with the nearly complete dissociation of the intact α_2M molecule to a species migrating as an α_2M subunit in gel electrophoresis, under both denaturing and nondenaturing conditions. The dissociation was accompanied by only small changes of the spectroscopic properties of the subunits, which thus retain a near-native conformation. Reaction of isolated subunits with methylamine or trypsin led to the appearance of ~0.55 mol of thiol group/mol of subunits, indicating that the thio ester bonds are largely intact. Moreover, the rate of cleavage of these bonds by methylamine was similar to that in the whole α_2M molecule. Although the bait region was specifically cleaved by nonstoichiometric amounts of trypsin, the isolated subunits had minimal proteinase binding ability. Reaction of subunits with methylamine or trypsin produced changes of far-ultraviolet circular dichroism and near-ultraviolet absorption similar to those induced in the whole α_2M molecule, although in contrast with whole α_2M no fluorescence change was observed. The methylamine- or trypsin-treated subunits reassociated to a tetrameric species, migrating as the "fast" form of whole α_2M in gradient gel electrophoresis. The subunits were eliminated from the circulation in the rat only slightly faster than intact α_2M. Reaction of the subunits with methylamine or trypsin increased the clearance rate somewhat, but this rate was still about 10-fold lower than that of trypsin-treated whole α_2M. These observations indicate that specific reduction of the interchain disulfide bonds of α_2M by thioredoxin leads to dissociation of the inhibitor into individual subunits, which retain many properties of intact α_2M but lack proteinase binding ability and the ability to fully expose the receptor recognition site on reaction with amines or proteinases.

α_2-Macroglobulin (α_2M)[1] is a large (M_r ~720 000) plasma proteinase inhibitor, containing four identical subunits,[2] that inhibits a wide variety of proteinases from all four subclasses (Jones et al., 1972; Barrett & Starkey, 1973; Harpel, 1976; Hall & Roberts, 1978). The target proteinase cleaves an exposed region of the polypeptide chain of α_2M, the bait region, and is subsequently trapped by a conformational change of the inhibitor in such a manner that it retains activity against low molecular weight, but not against high molecular weight, substrates (Harpel, 1973; Barrett & Starkey, 1973; Barrett et al., 1979; Swenson & Howard, 1979; Sottrup-Jensen et al., 1981b; Gonias et al., 1982; Björk & Fish, 1982; Dangott et al., 1983). The conformational change also leads to exposure of a receptor recognition site involved in uptake of the α_2M–proteinase complex into cells (Debanne et al., 1976; Van Leuven et al., 1979, 1986; Kaplan et al., 1981; Marynen et al., 1981; Imber & Pizzo, 1981). An α_2M molecule can maximally bind two molecules of proteinase, suggesting that the half-molecule is the functional unit of the inhibitor (Ganrot, 1966; Barrett et al., 1979; Swenson & Howard, 1979; Sottrup-Jensen et al., 1980; Gonias & Pizzo, 1983; Björk et al., 1984). Each subunit of α_2M contains a thio ester bond between a cysteine residue and a neighboring glutamic acid residue (Sottrup-Jensen et al., 1980, 1981a; Howard, 1981; Salvesen et al., 1981). This bond is cleaved on reaction with proteinases, liberating a thiol group and covalently linking some enzyme molecules to the inhibitor via the glutamic acid residue (Salvesen & Barrett, 1980; Salvesen et al., 1981; Sottrup-Jensen et al., 1981c; Wu et al., 1981). Small primary amines

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[1] Abbreviations: α_2M, α_2-macroglobulin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; STI, soybean trypsin inhibitor; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

[2] Throughout the paper, the term "subunit" is used to denote the individual chains of α_2M. This term was selected in preference to the term "polypeptide chain" to indicate that the units are obtained by mere reduction of the interchain disulfide bonds with minimal unfolding of their native structure. The term "monomer" has not been used, since the intact α_2M molecule easily aggregates into polymers, in which reaction the whole molecule would appropriately be designated as a monomer.

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also cleave the thio ester bonds, leading to inactivation of human α_2M via a series of conformational changes, the final state of conformation being very similar to that induced by the reaction of the inhibitor with proteinases (Barrett et al., 1979; Björk & Fish, 1982; Gonias et al., 1982; Dangott et al., 1983; Larsson & Björk, 1984; Strickland & Bhattacharya, 1984; Larsson et al., 1985, 1987).

The four subunits of α_2M are linked pairwise by two interchain disulfide bonds in each subunit pair (Jones et al., 1972; Harpel, 1973; Hyldgaard Jensen & Sottrup-Jensen, 1986). Two such dimeric units of covalently joined subunits are associated into the functional, tetrameric structure by noncovalent interactions (Jones et al., 1972; Hall & Roberts, 1978; Barrett et al., 1979). Limited reduction of α_2M with dithiothreitol, which cleaves the interchain disulfide bonds but also several intrachain bonds, has been reported to produce free subunits (Barrett et al., 1979) or half-molecules of α_2M consisting of two noncovalently associated subunits (Gonias & Pizzo, 1983). The small redox protein thioredoxin and the associated enzyme thioredoxin reductase constitute a thiol-dependent reduction–oxidation system that can catalyze the reduction of certain protein disulfides by NADPH, usually with high selectivity (Holmgren, 1984, 1985). In this report, we show that the thioredoxin system can specifically reduce the interchain disulfide bonds of human α_2M with minimal cleavage of intrachain bonds. This reaction results in dissociation of the protein into free subunits, the properties of which are described.

Materials and Methods

α_2M was isolated from human plasma and β-trypsin (EC 3.4.21.4) from commercial bovine trypsin (type III; Sigma Chemical Co., St. Louis, MO), as described previously (Björk & Fish, 1982; Björk et al., 1985). Active-site titrations of the enzyme with 4-nitrophenyl 4-guanidinobenzoate (E. Merck, Darmstadt, West Germany; Chase & Shaw, 1970) gave 0.83 ± 0.01 (SD, n = 3) mol of active sites/mol of protein. Thioredoxin and thioredoxin reductase were purified by procedures described previously (Holmgren & Reichard, 1967; Thelander, 1967) from the *Escherichia coli* strain SK3981. This strain contains a plasmid, into which a fragment of *E. coli* DNA containing the thioredoxin gene has been cloned (Holmgren, 1985). Alternatively, the two proteins were obtained from IMCO Corp. Ltd, Stockholm, Sweden.

Disulfide bonds in α_2M (12 μM in 0.05 M Hepes/NaOH, 0.1 M NaCl, and 1 mM EDTA, pH 7.5) were reduced at 25 °C with 50 μM thioredoxin, 0.2 μM thioredoxin reductase, and 0.4 mM NADPH in a total volume of 500 μL. The time course of the reaction was monitored in a Zeiss PMQ3 spectrophotometer (Zeiss, Oberkochen, West Germany) or in a Cary 219 spectrophotometer (Varian Instruments, Palo Alto, CA) by the decrease in the absorbance of NADPH. A molar absorption coefficient of 6200 M^{-1} cm^{-1} at 340 nm was used to calculate the number of disulfide bonds reduced (Holmgren, 1984). At the end of the experiment, iodoacetamide, or in some cases iodoacetate, was added to a concentration of 3 mM in order to block the thiol groups formed. Iodo[^{3}H]acetate (specific activity 2150 GBq/mol; Amersham International, Amersham, Bucks, U.K.) was used when radioactive labeling of the thiol groups was desired. After 10 min, the sample was applied to a Sepharose 6B column (1.9 × 30 cm; Pharmacia, Uppsala, Sweden), eluted at a flow rate of 50 mL/h. The peak containing subunits was concentrated in an SM-13200 collodion bag (Sartorius, Göttingen, West Germany).

Polyacrylamide gradient gel electrophoresis was performed on 4–30% gels (Pharmacia; Björk, 1985). Before analyses of mixtures of α_2M or α_2M subunits with trypsin, the enzyme was inactivated for 15 min at 25 °C with 2.5 mM TLCK. About 15 μg of protein was applied to each well. Marker proteins with molecular weights in the range 67 000–669 000 (Pharmacia) were run concurrently. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was done on 5% gels (Weber & Osborn, 1969). In analyses of bait region cleavage by trypsin, the enzyme was inactivated as described previously (Björk et al., 1984, 1985). Amounts of ~25 μg of protein were loaded on each gel.

The stoichiometry and kinetics of thiol group appearance in the reaction of α_2M or α_2M subunits with methylamine were analyzed by a continuous spectrophotometric assay, in which these groups were allowed to react with 5,5′-dithiobis(2-nitrobenzoic acid) (Larsson & Björk, 1984). The number of thiol groups released in the reaction with trypsin was measured by a similar method (Björk et al., 1984).

The inhibitory effect of α_2M subunits on the action of trypsin on high molecular weight substrates was measured by assays in which a fibrin gel (Björk et al., 1984) or hide powder azure (Barrett et al., 1979) was used as substrate for the enzyme. The ability of the subunits to protect trypsin from inactivation by STI (Ganrot, 1966) was analyzed with a chromogenic trypsin substrate (Björk et al., 1984). The binding of ^{125}I-labeled trypsin to α_2M subunits was determined by gel chromatography (Björk, 1985). Before the sample was applied to the column, TLCK, or, in some experiments STI was added to a concentration of 400 or 75 μM, respectively.

Ultraviolet absorption difference spectra, circular dichroism spectra in the near- and far-ultraviolet wavelength regions, and tryptophan fluorescense emission spectra of α_2M or α_2M subunits with or without methylamine or trypsin were measured as detailed in previous publications (Björk & Fish, 1982; Björk et al., 1985). The kinetics of the absorption changes accompanying the reaction with methylamine were monitored by difference measurements, as described earlier (Larsson et al., 1985).

For analyses of the survival of intravenously injected proteins in the circulation of the rat, α_2M was labeled with ^{125}I with the Enzymobead reagent (Bio-Rad, Richmond, CA) to a specific activity of ~7 × 10^3 cpm/μg. The radiolabeled α_2M, or subunits prepared from it, was reacted with methylamine or trypsin, and TLCK was then added to all samples to a concentration of 2 mM. Male Sprague-Dawley rats (Anticimex, Stockholm, Sweden), weighing approximately 200 g, were anesthetized by intraperitoneal administration of pentobarbital (ACO, Stockholm, Sweden), and a 600-μL sample, containing ~1 mg of protein, was injected through the tail. Blood radioactivity was monitored by repeated withdrawal of 25-μL blood samples from the tail tip with calibrated capillary tubes. Control experiments with native and methylamine-treated α_2M with or without the addition of TLCK gave identical results.

All reactions of α_2M or α_2M subunits with methylamine, except the kinetic studies, were done for 2 h at 25 °C with 200 mM amine. The solvent in most reactions with the amine was 0.05 M Hepes/NaOH, 0.1 M NaCl, and 1 mM EDTA, pH 8.0. However, 0.2 M Hepes/NaOH, 0.1 M NaCl, and 1 mM EDTA, pH 8.00, was used in the studies of the kinetics of thiol group appearance and absorbance change to ensure a constant pH (Larsson & Björk, 1984), and 0.05 M Tris-HCl, 0.1 M NaCl, and 1 mM EDTA, pH 8.0, had to be used in the far-ultraviolet circular dichroism analyses because of the absorbance of Hepes in this wavelength region. Reactions with trypsin were done for 5 min at 25 °C with molar ratios of

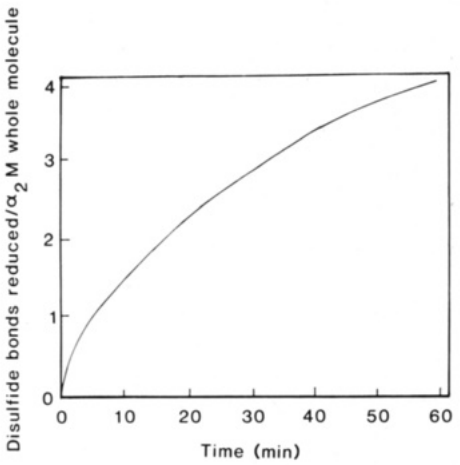

FIGURE 1: Time dependence of disulfide bond reduction, monitored by the absorbance decrease due to the consumption of NADPH, in the reaction of α_2M with the thioredoxin system. The reaction conditions are described under Materials and Methods.

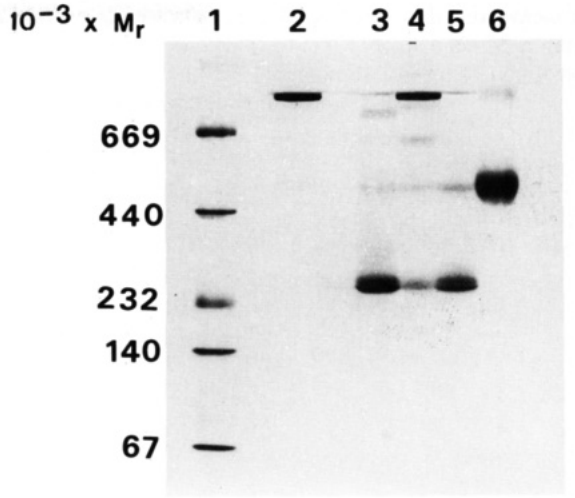

FIGURE 2: Gradient gel electrophoresis of products of reduction or dissociation of α_2M by different methods. Lane 1, marker proteins with the molecular weights indicated; lane 2, untreated α_2M; lane 3, α_2M reduced for 60 min with the thioredoxin system under the conditions described under Materials and Methods; lane 4, α_2M (6.4 μM) reduced for 3 h with 10 mM *N*-acetylcysteine in 0.05 M sodium phosphate, pH 8.0, conditions shown by Jones et al. (1972) to produce free subunits; lane 5, α_2M (2.0 μM) reduced for 30 min with 1 mM DTT in 0.05 M Tris-HCl, pH 8.1, conditions shown by Barrett et al. (1979) to give free subunits; lane 6, α_2M (2.0 μM) treated for 6 h with 4 M urea in 0.05 M Tris-HCl, pH 8.1, producing half-molecules consisting of two disulfide-linked subunits (Jones et al., 1972; Barrett et al., 1979). Thioredoxin or thioredoxin reductase is not visible in lane 3, due to its small size and low concentration, respectively.

trypsin to whole α_2M of 2.2:1 and of trypsin to α_2M subunits of 1.1:1, except where stated otherwise. When reactions with methylamine and trypsin were compared, the reactions with the enzyme were done in the same buffer as the methylamine reactions, i.e., at pH 8.0. In all other reactions with trypsin, the solvent was 0.05 M Hepes/NaOH, 0.1 M NaCl, and 1 mM EDTA, pH 7.5.

Protein concentrations were obtained by absorption measurements at 280 nm. The specific absorption coefficients (in liters per gram per centimeter) and molecular weights used in the calculations were 0.90 and 720000, respectively, for α_2M (Dunn & Spiro, 1967; Jones et al., 1972; Hall & Roberts, 1978; Sottrup-Jensen et al., 1984), 0.90 (i.e., the same as for α_2M) and 180000, respectively, for α_2M subunits (Sottrup-Jensen et al., 1984), 1.54 and 23300, respectively, for trypsin (Robinson et al., 1971; Walsh & Neurath, 1964), 1.01 and 20100, respectively, for STI (Yamamoto & Ikenkar, 1967; Koide & Ikenaka, 1973), 1.14 and 12000, respectively, for thioredoxin (Laurent et al., 1964; Holmgren & Reichard, 1967), and 1.40 and 70000, respectively, for thioredoxin reductase (Thelander, 1967; Williams, 1976). Trypsin concentrations were active-site concentrations.

Results

Reduction of α_2M with the Thioredoxin System and Identification of the Major Reaction Product as Free Subunits. The time course of the reaction between the thioredoxin system and α_2M was analyzed by the consumption of NADPH, reflecting disulfide bond cleavage (Holmgren, 1984), and by gradient gel electrophoresis. Reaction for 60 min under the conditions selected resulted in the reduction of 4.1 ± 0.25 (SD, n = 3) disulfide bonds per α_2M molecule (Figure 1), approximately the value expected from cleavage of all four interchain disulfide bonds of α_2M (Hyldgaard Jensen & Sottrup-Jensen, 1986) but no intrachain bonds. This extent of disulfide bond reduction was found to coincide with the nearly complete conversion of the intact α_2M molecule to a species which behaved in gradient gel electrophoresis as having an apparent molecular weight of about 250000 (Figure 2). A minor band with an apparent molecular weight of about 500000 was also observed in all experiments, as was a minimal amount of intact α_2M in many cases. However, the faint band migrating as the "fast" form of α_2M (Barrett et al., 1979) in this particular analysis was only seen occasionally. The electrophoretic pattern was the same whether iodoacetamide or iodoacetate was used to block liberated thiol groups; iodoacetamide was used routinely. In sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions, α_2M that had been reduced with the thioredoxin system migrated similarly to subunits obtained by full reduction of α_2M in dodecyl sulfate, in support of the conclusion that all interchain disulfide bonds of the inhibitor had been cleaved by thioredoxin.

To establish the nature of the molecular species obtained by reduction of α_2M with the thioredoxin system, we compared these species by gradient gel electrophoresis with previously characterized products of reduction or dissociation of α_2M by other methods (Figure 2). Thus, the band with apparent M_r ~250000 migrated identically with free subunits shown earlier to be produced by reduction of α_2M with *N*-acetylcysteine (although only partial reduction and dissociation was obtained in our hands) or DTT under the conditions used (Jones et al., 1972; Barrett et al., 1979). Moreover, the minor band with apparent M_r ~500000 had the same mobility as half-molecules consisting of two disulfide-linked subunits, obtained by dissociation of α_2M by urea as described previously (Jones et al., 1972; Barrett et al., 1979). We thus conclude that the apparently specific reduction of the interchain bonds of α_2M by the thioredoxin system produces predominantly free subunits, with the concurrent appearance of a small amount of a molecular species behaving as half-molecules of α_2M. The sequel to this conclusion, i.e., that the apparent molecular weights estimated for these forms are anomalously high, is supported by the observation that also intact α_2M behaves in comparison with the marker proteins as a molecule with a larger hydrodynamic volume than expected from its molecular weight (Figure 2).

Reduction of Methylamine- and Trypsin-Treated α_2M. Reaction of methylamine- or trypsin-treated α_2M with the thioredoxin system under the conditions used for the untreated inhibitor resulted in the reduction of 9.8 and 9.4 disulfide bonds

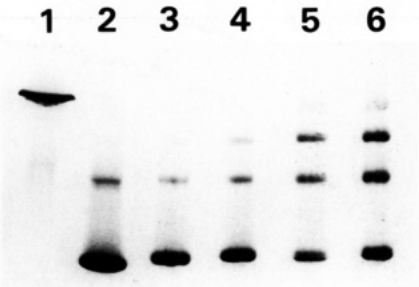

FIGURE 3: Analysis by gradient gel electrophoresis of reassociation of isolated α_2M subunits. Lane 1, α_2M; lane 2, α_2M reduced with the thioredoxin system; lane 3, subunits immediately after isolation by gel chromatography; lanes 4, 5, and 6, subunits after incubation at 25 °C for 2, 12, and 24 h, respectively.

per molecule of the two forms, respectively, in 60 min. After this reaction, maximally 5% of either form of the inhibitor had dissociated to free subunits, as shown by gradient gel electrophoresis. Moreover, no material migrating as individual subunits in sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions was detected. These observations indicate that the conformational changes accompanying the reaction of α_2M with methylamine or trypsin (Gonias et al., 1982; Björk & Fish, 1982; Larsson et al., 1987) render several intrachain disulfide bonds in each subunit easily accessible to reduction by thioredoxin but also cause the interchain bonds to become inaccessible to this reducing agent. Partially contrasting findings have been reported earlier for the less specific reduction of α_2M with DTT, which apparently cleaves the interchain disulfide bonds also in methylamine- and trypsin-treated α_2M, although the subunits remain associated (Barrett et al., 1979; Gonias & Pizzo, 1983).

Isolation and General Properties of Subunits. The free subunits produced by thioredoxin reduction were isolated by gel chromatography on Sepharose 6B after the liberated thiol groups had been blocked with iodoacetamide. The major peak eluting from the column, at K_{av} ~0.4, consisted predominantly of subunits (Figure 3). It appeared well separated from a smaller, trailing peak, which eluted slightly before the total volume of the column and contained the components of the thioredoxin system. In spite of judicious pooling and repeated gel chromatography of the subunit peak, we found it impossible to reduce the contamination of material migrating as half-molecules in gradient gel electrophoresis to a level below that shown in Figure 3. This inability was attributed to slow reassociation of the subunits to forms migrating as two-subunit, three-subunit, and even four-subunit species (Figure 3). The latter behaved in electrophoresis as the "slow", intact form of α_2M (Barrett et al., 1979). Due to this reassociation behavior, all experiments were done immediately after isolation of the subunits. The preparations used consisted of 90–95% subunits, as estimated from scanning of the gels.

Analyses in which thiol groups were blocked with iodo-[^{3}H]acetate after thioredoxin reduction of α_2M showed an incorporation of 1.72 ± 0.21 (SD, n = 3) mol of reagent/mol of isolated subunit. This value supports the conclusion from the analyses of NADPH consumption during the reduction that the two interchain disulfide bonds in each subunit pair (Hyldgaard Jensen & Sottrup-Jensen, 1986), but a minimal amount of intrachain bonds, were cleaved. Spectroscopic analyses showed small differences between intact α_2M and its isolated subunits. Thus, the far-ultraviolet circular dichroism spectrum of free subunits was slightly less negative than that of intact α_2M (Figure 4A). Moreover, the near-ultraviolet circular dichroism spectra of subunits and α_2M differed

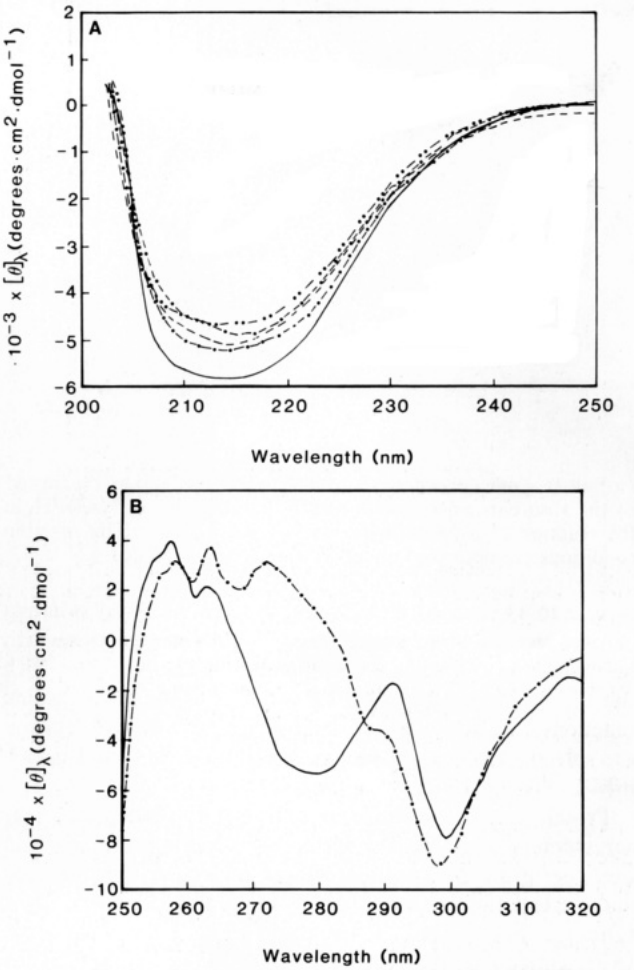

FIGURE 4: Circular dichroism spectra of α_2M and α_2M subunits before and after reaction with methylamine or trypsin. (—) α_2M; (---) α_2M reacted with trypsin; (-·-) α_2M subunits; (--·--) α_2M subunits reacted with methylamine; (-··-) α_2M subunits reacted with trypsin. (A) Far-ultraviolet wavelength region. Cells with 0.1-cm path lengths were used, and concentrations of α_2M and α_2M subunits were 0.5 and 2.0 μM, respectively (i.e., ~0.35 mg/mL). The unit on the ordinate is mean residue ellipticity. The spectrum for α_2M reacted with methylamine is omitted from the figure for the sake of clarity but was highly similar to that of α_2M reacted with trypsin (Björk & Fish, 1982). (B) Near-ultraviolet wavelength region. Cells with 1-cm path lengths were used, and concentrations of α_2M and α_2M subunits were 2.1 and 8.3 μM, respectively (i.e., ~1.5 mg/mL). The unit on the ordinate is molar ellipticity. The bandwidth was 2 nm in both (A) and (B).

somewhat, primarily in the wavelength region around 280 nm (Figure 4B). Also, reduction and dissociation of α_2M into subunits resulted in about a 25% increase in tryptophan fluorescence (Figure 5). These differences may have arisen merely from disruption of the interactions between the subunits with minimal changes of overall three-dimensional structure. However, they are also compatible with a limited conformational change of the subunits on reduction or dissociation.

Reaction of Subunits with Methylamine or Trypsin. Reaction of the isolated subunits with methylamine or trypsin released 0.53 ± 0.07 (SD, n = 4) and 0.52 ± 0.03 (n = 3) thiol groups/mol of subunit, respectively. An analogous release, reflecting thio ester bond cleavage (Sottrup-Jensen et al., 1980; Howard, 1981), of 0.8–0.9 thiol group/subunit was measured in whole α_2M. Treatment of the subunits with 4 M guanidinium chloride did not cause any appearance of thiol groups and did not appreciably increase the amount of such groups released by methylamine. The kinetics of thiol group appearance (Larsson & Björk, 1984) in α_2M and isolated sub-

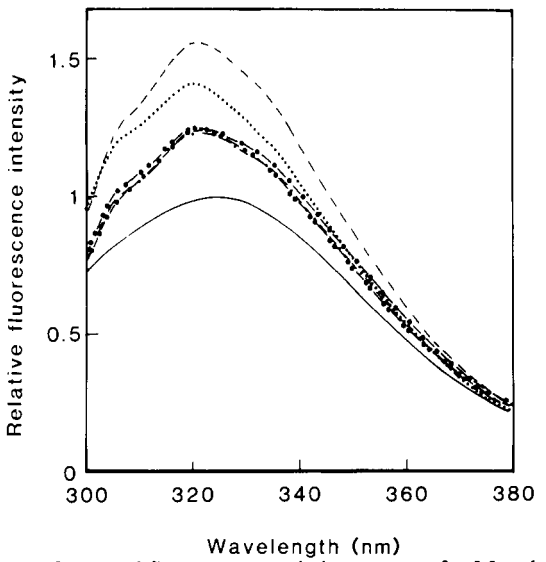

FIGURE 5: Corrected fluorescence emission spectra of α_2M and α_2M subunits before and after reaction with methylamine or trypsin. (—) α_2M; (···) α_2M reacted with methylamine; (- - -) α_2M reacted with trypsin; (—·—) α_2M subunits; (— — —) α_2M subunits reacted with methylamine; (—··—) α_2M subunits reacted with trypsin. The excitation wavelength was 280 nm, and the excitation and emission bandwidths were 8 and 2 nm, respectively. Concentrations of α_2M and α_2M subunits were ~0.14 and ~0.55 μM, respectively (i.e., 0.1 mg/mL). Inner filter corrections were done as described earlier (Björk & Fish, 1982). The spectrum for α_2M has been normalized to 1.0.

units on reaction with methylamine were similar (Figure 6). Second-order rate constants, based on the concentration of unprotonated amine (Larsson & Björk, 1984), of 18.4 and 17.0 M^{-1} s^{-1} for α_2M and 20.8 and 15.7 M^{-1} s^{-1} for subunits were obtained in the reactions with 100 and 200 mM methylamine, respectively, at pH 8.00.

The isolated subunits were found to bind a minimal amount of trypsin. The subunits thus inhibited <0.1 mol of trypsin/mol of subunit in the fibrin gel and hide powder azure assays (Figure 7) and protected only ~0.02 mol of trypsin/mol of subunit from inactivation by STI. Similarly, the subunits bound <0.1 mol of ^{125}I-labeled trypsin/mol of subunit, regardless of whether trypsin was inactivated with TLCK or STI before gel chromatography. Some of this inhibitory capacity may be due to possibly remaining intact α_2M in the preparations. Incubation of the subunits at 25 °C for up to 5 days did not result in an increased activity in the hide powder azure or STI assays, indicating that the reassociation of the subunits to molecular species containing two to four subunits (see above) does not lead to significant regaining of inhibitory activity. The isolated subunits were cleaved by trypsin to fragments identical with or highly similar to those produced by cleavage of the bait region in whole α_2M (Figure 8). However, in contrast with the latter reaction, less than stoichiometric amounts of enzyme were sufficient for this cleavage.

Reaction of the α_2M subunits with methylamine or trypsin resulted in a small increase of the far-ultraviolet circular dichroism spectrum that was of a similar magnitude as the increase induced in the spectrum of the whole α_2M molecule (Figure 4A). The circular dichroism changes in the near-ultraviolet wavelength region were not investigated, since reaction of α_2M with methylamine or trypsin only slightly perturbs the spectrum of the protein in this region (Björk & Fish, 1982). Difference spectra showed changes of the ultraviolet absorption of the subunits on reaction with methylamine that were highly similar to those induced in whole α_2M, while the corresponding changes caused by trypsin differed somewhat from, but nevertheless had large similarities with,

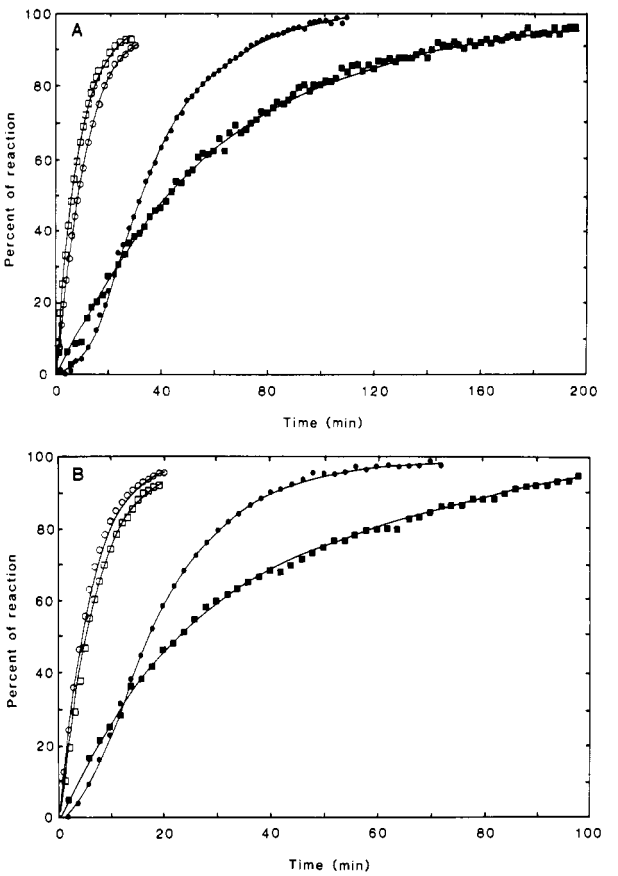

FIGURE 6: Kinetics of thiol group appearance and increase in ultraviolet absorption at 295 nm of α_2M and α_2M subunits in the reaction with methylamine. (A) 100 mM methylamine; (B) 200 mM methylamine. (○) thiol group appearance in α_2M; (●) increase in absorption of α_2M; (□) thiol group appearance in α_2M subunits; (■) increase in absorption of α_2M subunits. The concentrations of α_2M and α_2M subunits were 3.5 and 13.9 μM, respectively (i.e., ~2.5 mg/mL), in the analyses of thiol group appearance and 2.1 and 6.1 μM (i.e., 1.5 and 1.1 mg/mL), respectively, in the absorption difference measurements. The solid lines represent nonlinear least-squares fits of the data to first-order reactions for the thiol group appearance and the increase in absorption of α_2M subunits, and to model D of Larsson et al. (1985) for the increase in absorption of α_2M. To facilitate comparison between experiments, the data have been plotted as the percent of reaction, with the use of the value at infinite time for the property analyzed that was derived from the computer fits.

the changes occurring in the whole molecule (Figure 9). In contrast with these two analyses, no change in tryptophan fluorescence could be detected when the subunits were allowed to react with either the amine or the enzyme (Figure 5).

Kinetic studies showed that the ultraviolet absorbtion changes, monitored at 295 nm, of the subunits in the reaction with methylamine occurred appreciably slower than the thiol group release and also slower than the corresponding absorption changes of whole α_2M [Figure 6; see also Larsson et al. (1985)]. Moreover, in contrast with the latter reaction, no lag phase could be discerned in the reaction of subunits with the amine. Instead, the data could be reasonably well fitted to a first-order reaction with a rate constant about one-fifth that of the thiol group appearance (Figure 6). These observations indicate that the absorbance change of the subunits occurs as a result of one or more sequential reactions following cleavage of the thio ester bond, one of these steps being sufficiently slower than the other steps of the reaction sequence that it is primarily responsible for the observed approximately first-order rate of change of the absorbance.

Gradient gel electrophoresis showed that the subunits reassociated to a four-subunit species, migrating as the "fast"

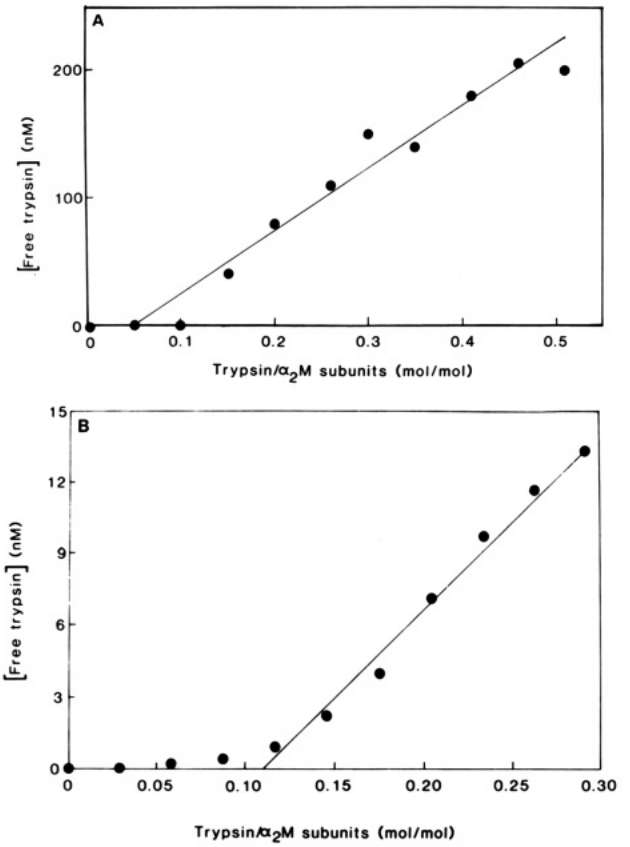

FIGURE 7: Inhibition of trypsin by α_2M subunits. (A) Fibrin gel assay; (B) hide powder azure assay. The assays were done as described by Björk et al. (1984) and Barrett et al. (1979), respectively. The concentration of α_2M subunits in the reaction with trypsin was 0.55 μM in (A) and 60 nM in (B).

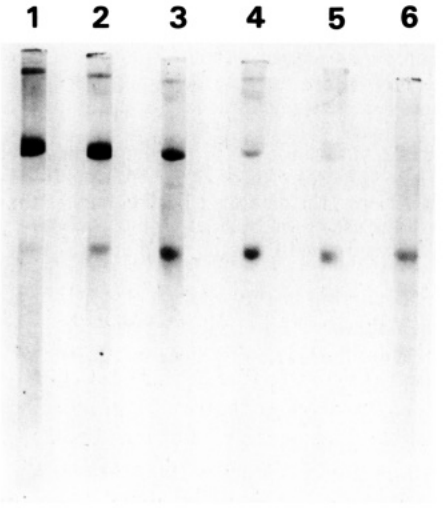

FIGURE 8: Trypsin cleavage of α_2M subunits, analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. Gel 1, α_2M subunits; gels 2–6, α_2M subunits reacted with trypsin in enzyme to inhibitor ratios of 0.03, 0.06, 0.11, 0.14, and 0.17, respectively. The analyses were done as described earlier (Björk et al., 1984, 1985). The concentration of α_2M subunits in the reaction with trypsin was 5.5 μM. The faster moving band seen on trypsin cleavage had the same mobility as the corresponding band (containing both fragments of the chain) obtained by reaction of whole α_2M with trypsin.

form of whole α_2M, on treatment with methylamine or trypsin (Figure 10). Thus, the changes induced by either reactant presumably result in the appearance of noncovalent interactions of appreciable strength between the subunits. Analogous

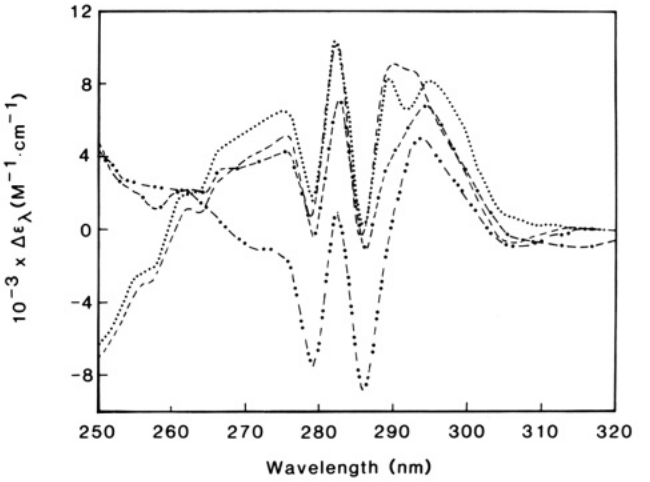

FIGURE 9: Ultraviolet absorbance difference spectra measured between α_2M or α_2M subunits reacted with methylamine or trypsin and untreated α_2M or α_2M subunits. (···) α_2M reacted with methylamine; (- - -) α_2M reacted with trypsin; (— · — · —) α_2M subunits reacted with methylamine; (— ··· —) α_2M subunits reacted with trypsin. Double-compartment cells with 1-cm path lengths per compartment were used, and concentrations of α_2M and α_2M subunits were 2.1 and 8.3 μM, respectively (i.e., ~1.5 mg/mL). The bandwidth was 1 nm.

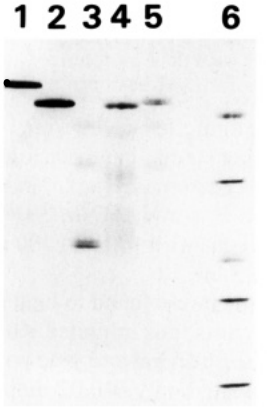

FIGURE 10: Gradient gel electrophoresis of α_2M and α_2M subunits before and after reaction with methylamine or trypsin. Lane 1, α_2M; lane 2, α_2M reacted with methylamine (reaction with trypsin gives identical mobility; Barrett et al., 1979); lane 3, α_2M subunits; lane 4, α_2M subunits reacted with trypsin; lane 5, α_2M subunits reacted with methylamine; lane 6, marker proteins (see Figure 1 for molecular weights).

reassociation was obtained with subunits or half-molecules produced by reduction of α_2M with DTT (Barrett et al., 1979; Gonias & Pizzo, 1983).

Survival of Subunits in the Circulation of the Rat. In agreement with previous results with other animal species (Ohlsson, 1971; Ohlsson & Laurell, 1976; Imber & Pizzo, 1981; Pizzo & Gonias, 1984), α_2M injected intravenously into the rat was slowly removed from the circulation ($t_{1/2} > 1$ h), whereas α_2M that had reacted with trypsin was cleared rapidly, with $t_{1/2}$ ~3 min (Figure 11). Methylamine-treated α_2M has been shown to be cleared with the same rapid rate as the trypsin-treated form (Imber & Pizzo, 1981; Pizzo & Gonias, 1984). Isolated subunits were eliminated slightly faster than intact α_2M ($t_{1/2} \sim 1$ h). Reaction of the subunits with methylamine or trypsin accelerated the uptake somewhat ($t_{1/2}$ ~30 min), but the rate of elimination was still far below that of trypsin-treated whole α_2M.

Discussion

These results show that the thioredoxin system can specifically reduce the four interchain disulfide bonds, two in each

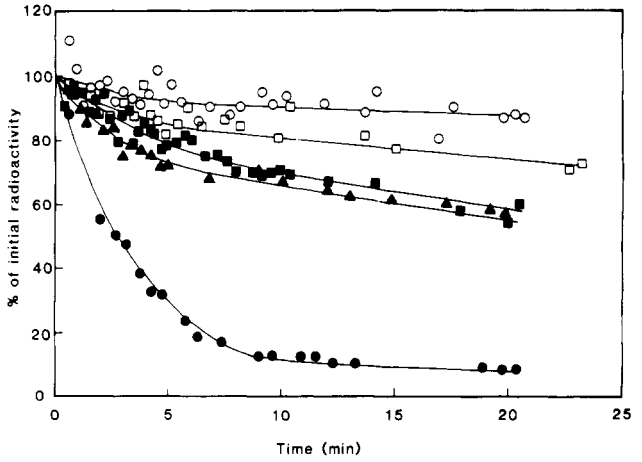

FIGURE 11: Circulatory survival in the rat of α_2M and α_2M subunits before and after reaction with methylamine or trypsin. (○) α_2M; (●) α_2M reacted with trypsin; (□) α_2M subunits; (■) α_2M subunits reacted with methylamine; (▲) α_2M subunits reacted with trypsin. For each experiment, the values are expressed as percent of the initial radioactivity obtained by extrapolation of the curves to zero time.

half-molecule (Hyldgaard Jensen & Sottrup-Jensen, 1986), of human α_2M, while minimally affecting the intrachain bonds. In previous work, the interchain bonds of native α_2M were cleaved by low molecular weight reducing agents, such as low concentrations of DTT, but this cleavage was accompanied by an additional reduction of one to three intrachain bonds per subunit (Barrett et al., 1979; Gonias & Pizzo, 1983). The larger size of the redox protein thioredoxin presumably ensures that essentially only the interchain bonds react. In the methylamine-treated form of α_2M, however, the intersubunit disulfide bonds apparently can be cleaved with a high degree of specificity by the low molecular weight reducing agent mercaptoethanesulfonate (Hyldgaard Jensen & Sottrup-Jensen, 1986).

This work furthermore shows that the α_2M molecule dissociates into free subunits following the specific reduction of the interchain disulfide bonds by thioredoxin. The appearance of a small amount of material behaving as half-molecules probably is due mainly to reassociation of separated subunits, since this process was shown to occur, although at a slow rate. In previous studies, reduction of α_2M with low molecular weight reducing agents has been reported to produce either free subunits (Jones et al., 1972; Barrett et al., 1979) or half-molecules containing two noncovalently bound subunits (Gonias & Pizzo, 1983; Sjöberg et al., 1985). The reason for these discrepant findings is unclear, especially in view of the similar conditions used. Our results are in disagreement only with the latter reports. Possibly, half-molecules were obtained in those studies due to the high extent of reduction of intrachain disulfide bonds caused by the low molecular weight reducing agent DTT. Cleavage of these bonds may have resulted in structural changes of the subunits that prevented dissociation of the α_2M molecule beyond the half-molecule stage. Alternatively, the half-molecules may have been formed by reassociation of free subunits. However, this possibility is less likely, since the half-molecules were reported to have proteinase binding ability, although lower than that of whole molecules (Gonias & Pizzo, 1983). In our work, no reappareance of such activity could be demonstrated on reassociation of separated subunits.

The isolated subunits obtained by thioredoxin reduction retain many properties of the whole α_2M molecule. The reduction and dissociation are accompanied by at most limited conformational changes of the subunits, which thus preserve a near-native conformation. Apparently, the noncovalent interactions between the subunits are disrupted by only minor structural perturbations induced by the disulfide bond cleavage. Comparison of the amount of thiol groups released by methylamine or trypsin in subunits and whole α_2M suggests that also the thio ester bonds are largely intact in the subunits. This conclusion is supported by the kinetics of thiol group appearance and by the spectroscopic changes, partially similar to those of the whole α_2M molecule, accompanying the reaction of subunits with methylamine. The somewhat lower amount of thiol groups released in the subunits than in whole α_2M may indicate that some thio esters were cleaved during the reduction and dissociation reaction. However, this possibility is not supported by the analyses of the thiol group content of the subunits, based on the incorporation of radioactive iodoacetate, which in fact showed a slightly lower amount of such groups than would be expected from cleavage of all interchain disulfide bonds. Alternatively, the reassociation of subunits following the reaction with methylamine or trypsin may trap some subunits with still intact thio ester bonds in such as manner that these bonds are refractory to cleavage. In such a case, however, the interactions preventing this cleavage must be very strong, since no additional thiol group release could be demonstrated on treatment of subunits with guanidinium chloride, with or without methylamine.

A further property of intact α_2M retained by the isolated subunits is that the bait region is accessible to cleavage by trypsin and presumably also other proteinases. Moreover, the subunits undergo a conformational change on reaction with the enzyme that has large similarities with the change occurring in whole α_2M, as judged by the spectral changes. The cleavage of the thio ester bonds in this reaction also indicates that a similar linkage between bait region proteolysis and thio ester bond cleavage exists in subunits as in the whole α_2M molecule. Nevertheless, the subunits bind no or a minimal amount of trypsin or, alternatively, bind the enzyme weakly with fully retained activity against high molecular weight substrates. An oligomeric arrangement of subunits thus presumably is necessary for the tight binding and inhibition of proteinases characteristic of the whole α_2M molecule. The association of two subunits to a half-molecule may provide most characteristics of this binding, as suggested by the well-established maximal inhibition of two proteinase molecules per whole molecule of α_2M (Ganrot, 1966; Barrett et al., 1979; Swenson & Howard, 1979; Sottrup-Jensen et al., 1980; Björk et al., 1984) and by the proteinase binding ability of separated half-molecules (Gonias & Pizzo, 1983). However, a specific interaction between the subunits is required for proteinase binding and inhibition, since random reassociation of isolated subunits to oligomeric structures does not result in the appearance of inhibitory activity. A manner in which such a specific assembly of subunits to an active molecule may occur is suggested in a recent model of α_2M structure and function (Feldman et al., 1985).

The conformational change of the subunits on reaction with methylamine or trypsin is reflected in changes of ultraviolet absorption and circular dichroism, whereas no change of tryptophan fluorescence is detectable. These observations may relate to the sequence of conformational changes occurring on reaction of the whole α_2M molecule with amines (Larsson et al., 1985). Thus, two sequential conformational changes can be distinguished in this reaction, the first of these resulting in an increase of tryptophan fluorescence without a concomitant absorbance change, and the subsequent change affecting the absorbance but not the fluorescence of the protein. Since

the fluorescence increase is not observed in the reaction of methylamine with free subunits, this change may primarily arise from an initial structural perturbation of the contact region between the subunits in the reaction of the amine with whole α_2M. The subsequent change of absorbance in this reaction may result from later changes of protein structure occurring preferentially within the subunits, as is also indicated by the kinetics of the absorbance change of isolated subunits. However, this interpretation, as well as a more detailed interpretation of the kinetics of the absorbance change, is complicated by the reassociation of the subunits occurring on reaction with the amine.

The finding that the isolated subunits are removed from the circulation in the rat slightly faster than intact α_2M is consistent with the receptor recognition site involved in the uptake of α_2M–proteinase complexes into cells (Debanne et al., 1976; Van Leuven et al., 1979, 1986; Imber & Pizzo, 1981) being exposed to some extent in the subunits. This finding is thus in general agreement with the model for α_2M structure and function proposed by Feldman et al. (1985), which predicts partial exposure of the receptor recognition site on dissociation of α_2M into individual subunits. In contrast with the results obtained for whole α_2M or half-molecules, reaction with methylamine or trypsin only slightly increases the rate of elimination of the subunits from the circulation. The receptor binding domain thus presumably is not fully exposed in the subunits by these reactions, as it is in the whole α_2M or half-molecules. This behavior may be due to somewhat different conformational changes in this region on reaction with amines or proteinases when the subunits are free or bound in whole α_2M. This proposal is consistent with the observed differences in spectroscopic changes. Alternatively, reaction of subunits with methylamine or trypsin does expose the receptor recognition site, but this site is partially blocked by the subsequent reassociation of the subunits.

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Biologically Active Fluorescent Derivatives of Spinach Calmodulin That Report Calmodulin Target Protein Binding†

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ABSTRACT: Spinach calmodulin (CaM) has been labeled at cysteine-26 with the sulfhydryl-selective probe 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS) to produce MIANS-CaM. The interaction of MIANS-CaM with CaM binding proteins was studied by fluorescence enhancement accompanying the protein–protein interactions. MIANS-CaM bound to smooth muscle myosin light-chain kinase with a K_d of 9 nM, causing a 4.6-fold fluorescence enhancement. Caldesmon bound with a K_d of 250 nM, causing a 2-fold fluorescence enhancement. Calcineurin (CaN) bound to MIANS-CaM with a K_d <5 nM, causing an 80% increase in fluorescence. On the other hand, binding of the CaM antagonist drugs prenylamine and calmidazolium or the potent peptide antagonist melittin did not alter MIANS fluorescence. MIANS-CaM activated brain cGMP phosphodiesterase and CaN as effectively as unlabeled CaM. Spinach CaM was also labeled with three other sulfhydryl reagents, 6-acryloyl-2-(dimethylamino)naphthalene, (2,5-dimethoxy-4-stilbenyl)maleimide, and rhodamine X maleimide. CaN bound to the highly fluorescent rhodamine X maleimidyl-CaM with a K_d of 1.4 nM, causing a 25% increase in polarization. Both MIANS-CaM and rhodamine X-CaM were used to monitor the Ca^{2+} dependence of the interaction between CaM and CaN. Half-maximal binding occurred at pCa 6.7–6.8 in the absence of Mg^{2+}, or at pCa 6.3 in the presence of 3 mM Mg^{2+}. In both cases, the dependence of the interaction was cooperative with respect to Ca^{2+} (Hill coefficients of 1.7–2.0). Use of these fluorescent CaMs should allow accurate monitoring of CaM interactions with its target proteins and perhaps their localization within the cell.

Calmodulin (CaM)[1] is a highly conserved protein which interacts with a large number of target proteins in a Ca^{2+}-dependent manner [for reviews, see Johnson and Mills (1986), Cox et al. (1984), and Manalan and Klee (1984)]. Vertebrate CaM contains no cysteine, no tryptophan, and two tyrosine residues (Dedman et al., 1977). In order to study the binding of CaM to target proteins having dissociation constants in the nanomolar range, it is necessary to attach highly fluorescent labels to the CaM. However, since vertebrate CaM lacks cysteine, it is difficult to achieve such labeling in a specific manner. IAEDANS has been used to label vertebrate CaM at methionines (Olwin et al., 1984), but it was found necessary to affinity purify the fluorescently labeled CaM to obtain reliable results since small amounts of unlabeled CaM markedly interfered with kinetic determinations. DANS-CaM has been used to monitor interactions with CaM's target proteins but was not sufficiently fluorescent to determine dissociation constant in the low nanomolar range and its fluorescence was affected by Ca^{2+} and CaM antagonist drugs (Kincaid et al., 1982; Malencik & Anderson, 1982; Johnson & Wittenauer, 1983). Spinach CaM has recently been sequenced and shown to contain a single cysteine residue at position 26 (Lukas et al., 1984). Since it appears to activate some target proteins in a manner identical with vertebrate CaM (Watterson et al., 1980), it represents an ideal candidate for specific fluorescent labeling with sulfhydryl-selective fluorescent probes.

In this paper, we describe the preparation and properties of 2-(4-maleimidoanilino)naphthalene-6-sulfonate (MIANS) spinach CaM and its interactions with three target proteins: smooth muscle myosin light-chain kinase, smooth muscle

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[1] Abbreviations: MOPS, 4-morpholinepropanesulfonic acid; CaM, calmodulin; MLCK, chicken gizzard smooth myosin light-chain kinase; CaD, caldesmon; PDE, phosphodiesterase; CaN, calcineurin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; App(NH)p, 5′-adenylyl imidodiphosphate; MIANS-CaM, 2-(4-maleimidoanilino)-naphthalene-6-sulfonic acid derivatized calmodulin; DANS-CaM, 5-(dimethylamino)naphthalene-1-sulfonyl chloride derivatized calmodulin; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)-aminomethane.

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