Conformational Changes Induced in Human α_2-Macroglobulin by Protease and Nucleophilic Modification†

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ABSTRACT: Human α_2-macroglobulin is a tetrameric protein of M_r 725000 that interacts with proteases and primary amines. The conformational changes associated with α_2-macroglobulin interaction with the natural substrate thrombin, other proteases, and methylamine were studied by using circular dichroism (205–350 nm), difference absorption spectroscopy (240–350 nm), and stopped-flow fluorescence spectroscopy. The far-ultraviolet circular dichroic spectrum of native α_2-macroglobulin was characterized by a negative band at 215 nm, with shoulders at 210 and 221 ± 1 nm, and is consistent with the presence of considerable ordered secondary structure. Reaction of the protein with proteases and methylamine leads to a small reduction in ellipticity between 205 and 230 nm. The absorption difference spectra of protease- and methylamine-reacted α_2-macroglobulin are similar in many respects, but some significant differences were noted. Protease-modified α_2-macroglobulin exhibited major difference extrema at 282.5 and 293 nm, assigned to tryptophan, with shoulders at 289.5 and 296 nm. The position and magnitude of the 293-nm difference band are consistent with the perturbation of about 5.4 ± 1.6 tryptophanyl residues in thrombin-reacted α_2-macroglobulin. The nucleophile-converted protein is missing the 293-nm difference peak, and the 296-nm component is well resolved. The 289.5- and 296-nm bands are unusual, and firm assignments cannot be made. The kinetics of conformational change of protease- and methylamine-modified α_2-macroglobulin were followed by difference spectroscopy at 282 nm for thrombin (37 °C) and methylamine (25 °C) and by stopped-flow fluorescence spectroscopy for trypsin (23 °C). The data for both thrombin- and methylamine-activated conformational changes in α_2-macroglobulin follow apparent first-order kinetics. The rate constants were 1.6×10^{-3} s^{-1} for thrombin (apparent first order at 2 mol of thrombin/mol of α_2-macroglobulin) and 5.1×10^{-4} s^{-1} for methylamine (first order). In contrast, trypsin-induced conformational changes appear to be at least biphasic with rate constants of 3.1×10^{-1} s^{-1} (rapid phase) and 1.84 s^{-1} (slow phase). The apparent first-order reaction of thrombin with α_2-macroglobulin is unexpected and unexplained but may reflect an early rate-limiting step not present in the reaction of α_2-macroglobulin with trypsin. Overall, these results suggest that the conformational changes induced in α_2-macroglobulin by reaction with proteases and methylamine are surprisingly similar and relatively limited in nature. Lastly, there are striking similarities in these physical indices of conformational changes in α_2-macroglobulin and those of another thio ester containing protein, the fourth component of complement [Isenman, D. E., & Kells, D. I. C. (1982) *Biochemistry 21*, 1109–1117].

α_2-Macroglobulin (α_2M)[1] is a broad specificity protease inhibitor found in plasma and has been suggested to be a growth factor for mouse embryonal carcinoma cells (Salomon et al., 1982). Human α_2M consists of four apparently identical polypeptide chains with molecular weights of approximately 185000 (Swenson & Howard, 1979a). Interaction of the inhibitor with proteases results in the cleavage of some or all of the macroglobulin subunits in a so-called "bait" region to yield major fragments of approximately 90000 molecular weight (Hall & Roberts, 1978). This reaction with proteases is believed to cause a shape change in α_2M, "entrapping" the protease(s) and sterically inhibiting the enzymic activity toward high molecular weight substrates (Barrett et al., 1974). A change in quaternary structure results (Bloth et al., 1968; Barrett et al., 1979) which may involve the shifting of the α_2M subunits from a square arrangement to a more compact tetrahedral form (Barrett et al., 1979). Evidence supporting this suggestion includes the increased relative mobility of the complex in nondenaturing electrophoretic gels, the "fast" electrophoretic form (Barrett et al., 1974), an apparent increase in isoelectric point (Van Leuven et al., 1981), changes in sedimentation behavior (Dangott & Cunningham, 1982; Gonias et al., 1982), and electron microscopy of the complexes (Bloth et al., 1968; Barrett et al., 1974).

In addition to the reaction with proteases, human α_2M has been shown to react with small primary amines and to then undergo a structural change which is similar to that induced by proteases (Barrett et al., 1979; Van Leuven et al., 1981; Bjork & Fish, 1982; Gonias et al., 1982). Reaction with an amine results directly in the cleavage of a putative internal thio ester bond in the subunits of α_2M, with covalent attachment of the amine to α_2M (Swenson & Howard, 1979b) and liberation of free sulfhydryl groups (Salvesen et al., 1981). This is in contrast to reaction with a protease which seems to involve first peptide bond hydrolysis in the bait region followed by reaction of the "trapped" protease or water with the thio ester. Similar conformations of amine- and protease-reacted α_2M are also suggested by reports that the two modified forms of the inhibitor, but not the unreacted form, compete for cell-surface receptors on fibroblasts (Van Leuven et al., 1978, 1979, 1981). These authors hypothesized that the shape change induced in α_2M creates or exposes the signal for receptor recognition and subsequent internalization of the complex.

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[1] Abbreviations: α_2M, α_2-macroglobulin; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; CD, circular dichroism; TPCK, tosylphenylalanyl chloromethyl ketone.

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The molecular properties of native α_2M have been examined by physical methods including ultracentrifugation, fluorescence, and circular dichroism (Jones et al., 1972; Frenoy et al., 1977; Richman & Verpoorte, 1981; Dangott & Cunningham, 1982; Bjork & Fish, 1982; Gonias et al., 1982). Triplet probe depolarization measurements (Pochon et al., 1978) and small-angle X-ray scattering studies of α_2M (Branegard et al., 1982) would suggest that only modest structural differences exist between the native and protease-bound molecule. Few studies have been performed, however, to characterize and compare the conformational rearrangement of α_2M after complex formation with proteases or inactivation with nucleophiles (Dangott & Cunningham, 1982; Bjork & Fish, 1982; Gonias et al., 1982).

Reorganization of subunits, which appears to be the basis of α_2M function, must be induced by conformational changes within the individual subunits. We have utilized circular dichroic and UV difference spectroscopy to characterize changes in α_2M conformation induced by reaction with thrombin, a natural ligand, and with other proteases and by nucleophilic inactivation. Our findings suggest an atypical, but ordered, secondary structure of α_2M as judged by current methods of analysis (Chang et al., 1978) and that there is only a relatively small change in this ordered structure between reacted and unreacted α_2M. Many characteristics of protease- and amine-reacted α_2M are strikingly similar (Bjork & Fish, 1982; Gonias et al., 1982) and indeed similar to those seen in the related thio ester containing complement protein C4 (Isenman & Kells, 1982), but unique differences can be detected. We have also been able, for the first time, to detect by stopped-flow fluorescence spectroscopy the relatively fast conformational change induced in α_2M by reaction with trypsin.

Materials and Methods

Purification of α_2M. Human α_2M was purified by Zn^{2+} chelate chromatography as previously described (Dangott & Cunningham, 1982). Each preparation of α_2M and α_2M complexes was monitored by nondenaturing gel electrophoresis and by $NaDodSO_4$–polyacrylamide gel electrophoresis. Nondenaturing polyacrylamide gel electrophoresis was performed in 5% acrylamide slabs (1.5 mm) according to Davis (1964). $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out in 7.5% slab gels according to Laemmli (1970). The samples for $NaDodSO_4$–polyacrylamide gel analysis were denatured in incubation buffer containing 2% $NaDodSO_4$, 5% β-mercaptoethanol, and 1 mM PMSF for 45 min at 37 °C. Myosin, β-galactosidase, phosphorylase *a*, bovine serum albumin, and ovalbumin were used as molecular weight standards. (Myosin was a gift of Dr. Dixie Frederiksen, Vanderbilt University. All other standards were purchased from Sigma Chemical Co., St. Louis, MO.) The gels were stained according to Fairbanks et al. (1971).

Modification of α_2M. All protease modification experiments were performed at a constant ratio of 2 mol of protease/mol of α_2M in 0.1 M sodium phosphate buffer (pH 8.0). Bovine trypsin (TPCK, 2× crystallized) was purchased from Worthington. Human thrombin was a generous gift of Dr. John W. Fenton, New York State Department of Health, Albany, NY. Protease concentrations were determined by active-site titration according to Chase & Shaw (1970). The concentration of α_2M was determined spectrophotometrically by using $E^{1\%,1cm}_{280nm} = 9.1$ (Dunn & Spiro, 1967) and $M_r = 725\,000$ (Jones et al., 1972). Human thrombin was reacted with the inhibitor for 1.5 h at 37 °C (Hall & Roberts, 1978). Trypsin was reacted with α_2M for 5 min at 25 °C. Nondenaturing polyacrylamide gel analysis of the complexes prepared in this manner indicated complete conversion of the α_2M to the "fast" electrophoretic form. $NaDodSO_4$–polyacrylamide gels of the α_2M–thrombin complexes showed that thrombin had apparently cleaved only about half of the α_2M subunits to polypeptides of approximately 90 000 molecular weight. On the other hand, trypsin apparently cleaved all the α_2M subunits to fragments of about the same molecular weight as those produced by thrombin proteolysis.

Studies of nucleophile-inactivated α_2M were performed by reacting the inhibitor with 0.1 M methylamine in 0.1 M phosphate buffer, pH 8.0, at 25 °C.

Spectral Measurements. The CD spectra were measured on a Cary 60 spectropolarimeter equipped with a CD attachment. Replicate scans were made with a full-scale sensitivity of 40×10^{-3} deg and a time constant of 3 s at ambient temperature. For wavelengths below 240 nm (0.5-mm cell), the α_2M concentration was 0.56 mg/mL, while above 240 nm (10-mm cell) the concentration was 1.75 mg/mL. CD spectra were collected on α_2M, the proteases, and complexes in 0.1 M sodium phosphate (pH 8.0) buffer. All buffers and protein solutions were prepared fresh and filtered with 0.2-μm Millipore filters which had been previously soaked in the pH 8.0 phosphate buffer.

Due to the fact that the proteases used are themselves optically active, an attempt was made to correct for their contribution. Separate spectra were recorded for the proteases under identical conditions in the absence of α_2M and their ellipticities arithmetically subtracted from the spectra of the complexes. All data are reported as ellipticity in degrees. When the data were reduced to mean residue ellipticity for purposes of comparison with reference spectra, an average residue molecular weight of 117.2 was used (Frenoy et al., 1977). In an attempt to facilitate comparison of reacted and unreacted α_2M, spectra were obtained on the same α_2M samples before and after reaction. For achievement of the latter, small volumes of concentrated perturbants were added directly to the cell, and the induced conformational change was allowed to go to completion. Added reactants caused less than 5% dilution of the protein sample. So that comparison of the various spectra could be facilitated, all scans presented were normalized to a standard α_2M concentration of 1 mg/mL. The extent of conversion was assessed by both nondenaturing and $NaDodSO_4$–polyacrylamide gel electrophoresis.

UV difference spectroscopy was performed on a Cary 210 spectrophotometer equipped with temperature-control cuvette towers. All difference spectra were obtained by using a matched pair of rectangular tandem cells (Hellma). One side of each tandem cell contained 1.0 mL of an α_2M solution (0.56 mg/mL in 0.1 M sodium phosphate buffer, pH 8.0) while the other side contained 1.0 mL of the appropriate reactant in the same buffer. A premixing base line was obtained and the reaction initiated in the sample cuvette by repeated inversion of cells. Temperature was controlled (25 ± 1 °C for experiments with methylamine and trypsin and 37 °C for thrombin) by the attachment of an external circulating temperature control bath. All scans were performed in duplicate.

Kinetic UV Difference and Fluorescence Measurements. UV difference rate measurements of the thrombin– and methylamine–α_2M reactions were monitored at a single wavelength (282 nm) with the Cary 210 spectrophotometer. The time course of the trypsin–α_2M reaction was monitored by fluorescence with a Durrum-Gibson stopped-flow spectrophotometer equipped with a Hewlett-Packard 1207B recording oscilloscope at ambient temperature. The excitation wave-

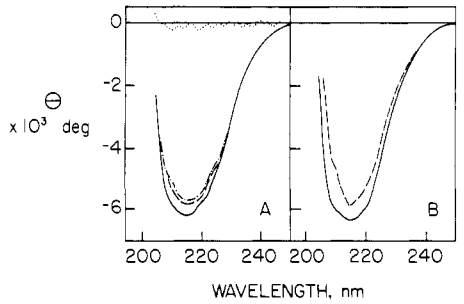

FIGURE 1: Far-UV CD spectra of reacted and unreacted human α_2M. (A) α_2M with human thrombin (molar ratio 1:2). (B) α_2M + 100 mM methylamine. (—) Unreacted α_2M; (---) α_2M-reacted complex; (···) reactant; (-·-) α_2M-reacted complex corrected for reactant. Data expressed as ellipticity in degrees for a 0.5-mm cell.

length used was 283 nm with a 4-nm excitation slit. The emission was monitored above 300 nm by using a Corning C.S. 0.54 filter.

Results

Circular Dichroism. The far-UV CD spectra of native human α_2M and α_2M reacted with human thrombin and methylamine are compared in Figure 1. Native α_2M exhibits a major negative band at approximately 215 nm, indicative of β structure, and shoulders at about 220–222 and 210 nm, indicative of α helicity (Chang et al., 1978). This is the first report on the CD spectrum of thrombin-reacted α_2M, while spectra of native and methylamine-reacted α_2M have been reported by others (Bjork & Fish, 1982; Gonias et al., 1982). Overall, the agreement is fairly good, but we were able to detect the 221 ± 1 nm shoulder in the α_2M CD spectra, which is important in the analysis of secondary structure. We also obtained the CD spectrum of bovine trypsin-reacted α_2M at 2 mol of trypsin/mol of α_2M (results not shown), and the spectrum was quite similar to that reported here for thrombin-reacted α_2M and reported by others for trypsin–α_2M complexes (Bjork & Fish, 1982; Gonias et al., 1982). Interestingly, Richman & Verpoorte (1981) found that at a 4:1 molar ratio of trypsin: α_2M the ellipticity of the complex was more negative than that of native α_2M between 203 and 240 nm.

There are numerous approaches for estimating the amounts of the various forms of ordered secondary structure in proteins. To analyze the α_2M CD spectrum, we have used the recent set of reference spectra for 15 proteins of known crystallographic structure (Chang et al., 1978). This approach involves a five-parameter solution,[2] namely, the fractions of α helix (f_α), β structure (f_β), β turns (f_t), and aperiodic structure (f_a) and the average number of residues per helix (n) (Chang et al., 1978). Using unconstrained analysis,[3] we obtained the following results, representing the best fit as judged by a sums test of f_i near 1.0, on the native α_2M CD spectrum after the data were reduced to the mean residue ellipticity: $f_\alpha = 0.33$, $f_\beta = 0.37$, $f_t = 0.15$, $f_a = 0.16$, and $n = 5.2$. The sum of the individual f_i's was 1.01, and the Pearson coefficient, obtained by comparing the experimental mean residue ellipticity with that calculated by using the above f_i and the reference spectra for each conformational term, was 0.996. Thus, the sums test and the Pearson coefficient indicate that an excellent fit to the reference spectra was achieved with the above parameters.

[2] The computer program for this analysis was kindly provided by James O. Gailit.

[3] The parameter f_i is the corresponding fraction of the individual structure elements; i.e., i can denote α helix, β structure, β turns, and aperiodic structure. The individual f_i's were not constrained to fall in the interval $0 \leq f_i \leq 1.0$ nor was f_i forced to be 1.0.

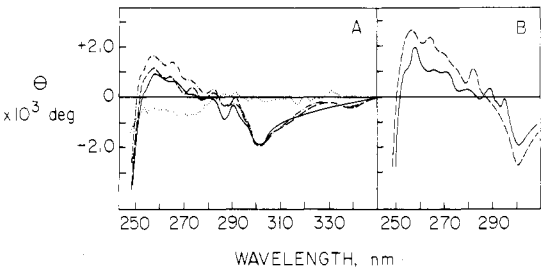

FIGURE 2: Near-UV CD spectra of reacted and unreacted human α_2M. (A) α_2M with thrombin (molar ratio 1:2). (B) α_2M with 100 mM methylamine. Symbols used are the same as those in Figure 1. Data expressed as ellipticity in degrees for a 10-mm cell.

Conversion of α_2M to a fast electrophoretic form by reaction with either thrombin or methylamine results in a less negative ellipticity below 230 nm. This change in ellipticity, which occurs in the presence of the nonchiral methylamine, could result from changes in f_i (e.g., an increase in f_t or a decrease in f_α and/or f_β) or n. However, the best fit to the data, again with the criterion that f_i be near 1.0, was achieved only when n was reduced to 4.3. The accompanying f_i values were within a few percent of those obtained for native α_2M with the exception of f_t which was reduced to 0.10 ($f_i = 0.99$). Whether this lower value reflects a real shortening of helix length or a perturbation of helical structure upon complex formation is difficult to resolve. The CD spectrum of thrombin-reacted α_2M was not analyzed since with two optically active components present one cannot unequivocally ascribe CD changes to α_2M. In point of fact, however, the CD spectrum of thrombin-reacted α_2M was almost identical with that of methylamine-reacted α_2M.

Near-UV CD spectra (Figure 2) of the native protein exhibit a complex series of weak positive extrema in the region 250–285 nm as well as a prominent negative band around 300–305 nm and extending to above 330 nm. The positive bands are in the wavelength region expected for the various aromatic side chains. The negative ellipticity between 300 and 350 nm is tentatively assigned to disulfide bonds. The unexpected and usually unreported ellipticity at these higher wavelengths was verified by recordings of a highly concentrated sample (12.9 mg/mL) of α_2M (data not shown). Reaction of the inhibitor with thrombin and with methylamine causes a general slight increase in positive ellipticity over the region 250–285 nm while methylamine leads to an enhancement of the negative ellipticity above 285 nm. These results are in general agreement with those of Bjork & Fish (1982) on trypsin- and chymotrypsin-reacted α_2M, although there are some differences in the two sets of spectra. We also obtained near-UV CD results on trypsin-reacted α_2M, and this spectrum was quite similar to that obtained with thrombin (data not shown).

UV Absorption Difference Spectroscopy. The UV absorption difference spectra produced by both proteolytic and nucleophilic conversion of α_2M are shown in Figure 3. The spectrum is shown for thrombin-reacted α_2M, but the spectra of trypsin- and α-chymotrypsin-reacted α_2M were almost identical (data not shown). The spectra shown in Figure 3 are similar, but there is a significant distinction. The difference spectrum for protease-modified α_2M exhibits two major peaks at 282.5 and 293 nm, as well as shoulders 289.5 nm and at approximately 296 nm. Nucleophilic conversion of α_2M results in a similar difference spectrum except that the major peak at 293 nm present in the protease-reacted protein is absent. Also, a major peak of difference absorbance is detected at approximately 296 nm which is tentatively identified with the

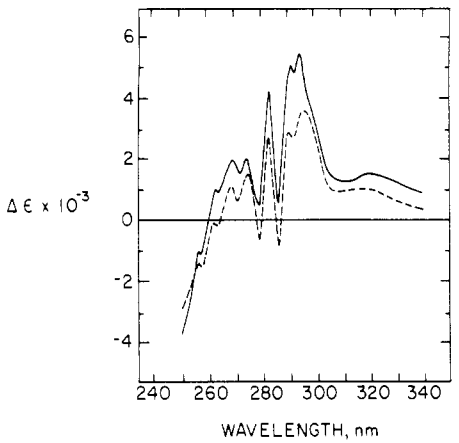

FIGURE 3: UV absorption difference spectra of α_2M modified with thrombin (—) and of α_2M modified with 0.1 M methylamine (- - -). Samples were allowed to temperature equilibrate in the light beam for 30 min prior to reaction. Scans were recorded in duplicate at a scan rate of 0.2 nm/s, a time constant of 1.0 s, and a full-scale deflection of 0.02 absorbance unit. Spectra were recorded over the region 235–450 nm and corrected for any mechanical shift. The solid and dashed curves were recorded at 1.5 and 2.5 h, respectively, after reaction. Ordinate values are expressed as M^{-1} cm^{-1}.

shoulder observed at this wavelength in the protease-induced difference spectrum. A broad positive extremum occurs around 320 nm and extends to about 385 nm in the spectra of both reactions. Several positive and negative extrema are observed between 250 and 275 nm. These spectra are similar to those reported by Bjork & Fish (1982) but are different from those presented by Gonias et al. (1982).

Time Course of Conformational Change Induced by Methylamine and Trypsin. Van Leuven et al. (1982), using gel electrophoretic analysis, have reported that the time course of conversion of α_2M to the fast electrophoretic form by methylamine inactivation is biphasic. We have used UV absorption difference spectroscopy at 282 nm to monitor the reaction (Table I, Figure 4B). Kinetic analysis of the reaction with 100 mM methylamine as first order yields a rate constant of about 5.1×10^{-4} s^{-1} and a $t_{1/2}$ of approximately 25 min. This result is in reasonable agreement with that of Gonias et al. (1982), obtained by kinetic analysis of far-UV CD spectral changes, who found with 50 mM methylamine a rate constant of 2.5×10^{-4} s^{-1} and a $t_{1/2}$ of 45 min. Samples of the reaction mixture taken at timed intervals for nondenaturing polyacrylamide gel analysis show conversion of the α_2M to the fast form at a rate consistent with the spectral data. Approximately 90 min are required for complete reaction under the conditions tested. However, as previously reported by Van Leuven et al. (1981), a small amount of α_2M (~10%) remains in the "slow" form even after 2 h of incubation with the methylamine.

Table I: Kinetic Constants for the Rate of Conformational Change of α_2-Macroglobulin by Proteases and Methylamine at pH 8.0

treatment	temp (°C)	k[a] (s^{-1})	$t_{1/2}$[b] (min)
Apparent Pseudo First Order			
methylamine	25	5.1×10^{-4} [c]	25
thrombin	37	1.6×10^{-3}	7
Biphasic			
trypsin fast phase	23	3.1×10^{-1}	4×10^{-2}
trypsin slow phase	23	1.84	3.7×10^{-1}

[a] The apparent rate constant k is calculated from the slope of log [α_2M] vs. time (seconds × −2.303). [b] $t_{1/2}$ is defined at 0.693/k. [c] With $n = 3$, the SD was $\pm 0.24 \times 10^{-4}$ s^{-1}.

Thrombin reaction with α_2M also lent itself successfully to spectroscopic measurement. Examination of the thrombin-induced conformational change suggests a $t_{1/2}$ of approximately 7 min (37 °C, pH 8.0) with a 2:1 molar ratio of thrombin to α_2M (Table I, Figure 4A). Complete conversion to the fast form requires about 30 min under the conditions tested. As indicated in Figure 4A, under the conditions used, this reaction could be characterized as apparent first order, although its complexity is well-known (Barrett & Starkey, 1973). Clearly, additional studies, including measurements at varying protease:α_2M ratios, are required to unequivocally ascertain the kinetic mechanism.

In order to follow the rate of conversion of α_2M by trypsin, we utilized stopped-flow fluorescence spectroscopy. Using standard fluorescence spectroscopy, it has been demonstrated that the intrinsic fluorescence of α_2M at 340 nm is increased by reaction with trypsin (Richman & Verpoorte, 1981; Straight & McKee, 1982). The $t_{1/2}$ of the change is approximately 0.5–1.0 s (Table I, Figure 4C).

Discussion

Several studies in recent years have attempted to elucidate the mechanism by which α_2-macroglobulin binds and inhibits proteases. The most frequently quoted and widely accepted hypothesis is that of Barrett et al. (1974) in which protease molecules are entrapped by the inhibitor after the macroglobulin undergoes a structural rearrangement. This hypothesis would seem to be consistent with several reported observations: the protection of entrapped proteases from the action of high molecular weight inhibitors but not low molecular weight inhibitors; the faster relative mobility of the

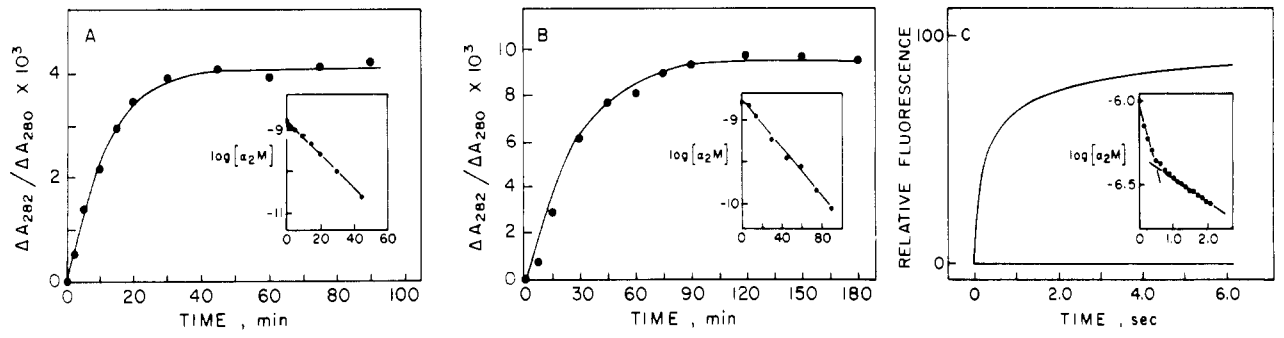

FIGURE 4: Kinetics of spectroscopic change induced by the addition of proteases and methylamine to human α_2M. (A) α_2M (0.5 mg/mL) and thrombin (2 mol of protease/mol of α_2M, 37 °C). (B) α_2M (0.56 mg/mL) and 0.1 M methylamine (25 °C). (C) α_2M (0.7 mg/mL) and trypsin (2 mol of protease/mol of α_2M, 23 °C). Continuous recording of conformational change as monitored by stopped-flow fluorescence emission. The excitation wavelength was 283 nm with a 4-mm excitation slit. The lower line represents the intrinsic fluorescence of native α_2M above 300 nm prior to reaction with the protease. Insets are first-order kinetic analyses of the reactions where log unreacted α_2M concentration (molar) is plotted vs. time. Straight lines were fitted to the data by least-squares analysis.

α_2M–protease complexes in native polyacrylamide gels; and the appearance of unreacted and reacted molecules in the electron microscope (Bloth et al., 1968; Barrett et al., 1974).

Most physical studies, however, have not been of sufficient resolution or design to detect the suggested shift in subunits from a planar to a tetragonal arrangement (Barrett et al., 1979). Rotational relaxation measurements (Frenoy et al., 1977) and low-angle X-ray scattering measurements of α_2M (Branegard et al., 1982) indicated that binding of protease to α_2M causes little change in the shape of the molecule. Also, Barrett et al. (1979) did not detect a change in the Stokes radius of α_2M upon binding α-chymotrypsin or inactivation by methylamine. However, in sedimentation velocity studies of fetal bovine α_2M, a small change in the frictional coefficient was found to result from trypsin binding (Dangott & Cunningham, 1982). This difference in the frictional coefficient is approximately that to be expected from a shift in the organization of tetrameric subunits from a planar to a tetrahedral form (Teller et al., 1979). Also, ultracentrifugal studies by Bjork & Fish (1982) and Gonias et al. (1982) suggest a 3–10% reduction in the Stokes radius as a result of the reaction of human α_2M with either trypsin or methylamine. Conformational changes within the subunits presumably induce this change in quaternary structure.

The far-UV CD spectrum of native α_2M appears to be unusual when compared to those for proteins of known structure and to those for synthetic homopolypeptides. We have attempted to obtained CD spectra of improved resolution and have used a more recent analysis in which average helix length and β turns are included in the estimation of secondary structure (Chang et al., 1978). We chose to use unconstrained analysis in order to avoid the potential dilemma introduced by forcing a poorly fit spectrum to conform to standard reference spectra. With this approach, the only way to satisfy the above conditions was with an average helix length of 5.2 residues. This is lower than the average experimental value of 10.4 found in 18 proteins of known crystallographic structure (Chang et al., 1978). In this context, it should be mentioned that average helical lengths, determined directly from X-ray-derived structures, are relatively low in several proteins of known structure, e.g., n = 4.0, 6.8, and 7.6 in concanavalin A, cytochrome c, and lysozyme, respectively (Chang et al., 1978). With these caveats, a conservative interpretation of our CD results is that the majority of the residues in α_2M are in some form of ordered secondary structure including appreciable α helicity. Furthermore, our estimate of β structure (37%) agrees well with that reported by others (Frenoy et al. 1977; Richman & Verpoorte, 1981; Bjork & Fish, 1982; Gonias et al., 1982), while our estimate of the α-helix content (33%) is higher than that previously suggested. This difference is explained in large part by our use of a revised standard spectrum for the α helix and our inclusion of the parameter n, the omission of which can lower the estimated helix content (Chen et al., 1972; Chang et al., 1978). The estimate that 15% of the residues in α_2M are involved in β turns is well within the range found from crystallographic studies of 18 proteins (Chang et al., 1978).

Reactions with proteases and with methylamine cause small, but reproducible, decreases in ellipticity between 205 and 230 nm, indicating that conformational changes accompany the methylamine or protease reaction. The reduced ellipticity of α_2M in the presence of proteases and methylamine does not, however, appear to arise from increased light scattering as judged by absorption spectra above 320 nm. At this point, the safest conclusion seems to be that the majority of the α_2M residues are distributed among the various types of ordered structure (although the average helix length appears atypical) and that only a modest conformational change accompanies the methylamine and the protease reaction.

Interpretation of the CD spectra in this region must also be made in light of recent observations (Holladay & Puett, 1976; Holladay et al., 1977) that aromatic residues, which may change the orientation or microenvironment during the conformational rearrangement, could contribute to the ellipticity over this region. Consistent with this reservation, we have detected difference spectra and small changes in the near-UV CD spectra of all α_2M complexes examined (Figure 2 and other unreported data) indicating changes in the microenvironments of some aromatic residue side chains. Whether these changes reflect alterations in intrasubunit or intersubunit interactions remains to be resolved. The presence of negative ellipticity persisting well above 300 nm suggests the contribution of disulfide chromophores to the spectrum (Kahn, 1979).

The involvement of specific aromatic residues in the conformational changes induced by thrombin and methylamine is reflected in the difference spectra. In contrast to the study of Gonias et al. (1982), several of the extrema present in our difference spectra of α_2M complexes are not readily assigned on the basis of known standards and results with other proteins. Two major features of the difference spectrum of the α_2M–thrombin complex are the peaks at 282.5 and 293 nm, which are assigned to tryptophanyl residues (Herskovits, 1967). The magnitude of the band at 293 nm in the α_2M–thrombin complex is consistent with, from three determinations, the perturbation of approximately 5.4 ± 1.6 (SD) tryptophan side chains[4] to more hydrophobic environments (Donovan, 1969). Assignment of the difference absorption bands at 289.5 and 296 nm are less easily made, however. The band 289.5 nm may be either an unusually red-shifted tyrosine or a blue-shifted tryptophan as the λ_{max} values for these two moieties are generally 286–288 and 292–294 nm, respectively (Herskovits, 1967). Gonias et al. (1982) have reported a tyrosine blue shift in spectra of α_2M complexes, suggesting exposure of several tyrosine residues to the aqueous environment. Our difference spectra appear to be better resolved and are more consistent with alterations in the environment of tryptophanyl groups. The position of the band we find at 296 nm is unlike any of the standard spectra reported or predicted by model compounds. The band may represent tryptophan side chains, as suggested by Bjork & Fish (1982), although they would be located in a very unusual hydrophobic environment.

Interpretation of the difference spectra of α_2M with thrombin and other proteases is complicated, as are the CD spectra, by the presence of the small amount of enzyme in the complex (~6.6% by weight). It is not possible a priori to calculate what contribution any perturbed residues within the protease molecule make to the total difference spectrum. Reaction with methylamine, on the other hand, should show contributions solely from α_2M since the amine is spectroscopically transparent in this region. It is both reassuring and interesting that all of the difference spectra show striking similarities in the overall pattern. However, the major absorption peak at 293 nm in α_2M–protease complexes, assigned to tryptophan, is absent from the amine-inactivated protein. The 293-nm tryptophan band is tentatively assigned to α_2M rather than to the protease since difference spectra of α_2M

[4] Interestingly, the trypsin-mediated conformational change leads to a value of 7.3 ± 1.6 (n = 4) tryptophans perturbed, but the difference relative to thrombin-mediated changes is not significant.

complexed with bovine trypsin and α-chymotrypsin are virtually superimposable upon that of the α_2M–thrombin complex. If this assignment is correct, then one or more tryptophan residues not perturbed by reaction with methylamine are perturbed by reaction with proteases. The major difference absorption maximum at 296 nm for α_2M–methylamine is similar to the shoulder present at the same wavelength in the protease-reacted inhibitor. The positive extremum at around 315 nm, seen in both reactions, is also unusual. It occurs over the region in which the CD spectra suggest disulfide contribution and may reflect the perturbation of specific disulfide groups during the conformational change.

The reaction of trypsin with α_2M, as followed by stopped-flow fluorescence, suggests the biphasic or even polyphasic reaction which might be anticipated in this complex system. In contrast, the rates of the change induced by methylamine and by thrombin can be described by apparent first-order kinetics monitoring ΔA at 282 nm. Similar rates were obtained by following the change of phenylalanyl band intensity at 274 nm (data not shown). The generally satisfactory description of the reaction of thrombin with α_2M by apparent first-order kinetics is surprising in view of the multimeric nature of α_2M and the low ratio of thrombin to α_2M. It may simply reflect the limitations of measurement under these particular conditions. Alternatively, it may suggest that α_2M readily binds thrombin but that the hydrolysis of the bait regions is rate limiting. More detailed kinetic studies at different concentrations are needed to address this question. The general conformity of our kinetic results from difference spectroscopy with those of Gonias et al. (1982), based on CD changes at 207 and 224 nm, strongly suggests that the change in conformation is a cooperative transition involving the peptide backbone and specific side chains simultaneously.

Lastly, similarities are noted between the conformational changes in α_2M and those which occur in the C4 component of human complement, another thio ester containing protein in which the conformation is dependent on the integrity of this unusual bond (Isenman & Kells, 1982). For example, the effects of methylamine on the CD spectra and the difference spectra of the two proteins are almost identical when our data are compared with those of Isenman & Kells (1982). The notable difference is that the difference spectral bands at 289.5 and 296 nm, observed in α_2M, are absent in C4 (Isenman & Kells, 1982). Moreover, in the three thio ester containing proteins which have been described, α_2M and the complement proteins C3 and C4, the amino acid sequences surrounding the putative thio ester sites are highly conserved (Swenson & Howard, 1980; Campbell et al., 1981; Thomas et al., 1982). This is of particular interest siince the subunit molecular weights and the oligomeric structures of α_2M and the complement proteins are quite different. Model building has shown that the proposed thio ester bond in the common sequence -Gly-Cys-Gly-Glu-Glu- is not compatible with these residues being in an α-helical conformation and a β turn could only occur if distorted (Tack et al., 1981; Thomas et al., 1982). We have applied the Chou–Fasman prediction rules (Chou & Fasman, 1978) to a 30-residue sequence containing the residues involved in the thio ester bond in α_2M and find only marginal probabilities for α helix, β structure or β turns. These preliminary results, in conjunction with model building (Tack et al., 1980), suggest that the amino acids in the region of the thio ester bond are probably not in any form of ordered secondary structure whether the thio ester bond is intact or cleaved. Thus, the methylamine-mediated conformational changes detected in these studies probably arise from changes in tertiary or quaternary structure triggered by cleavage of the thio ester bond, and not from changes in the secondary structure of the rather restricted region of the thio ester bond itself.

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Registry No. Trypsin, 9002-07-7; thrombin, 9002-04-4; methylamine, 74-89-5.

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Mechanism of Adenosinetriphosphatase Activity of Trinitrophenylated Myosin Subfragment 1†

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ABSTRACT: The rate and extent of conformational changes induced by nucleotides in trinitrophenylated myosin subfragment 1 (S-1) were studied. The absorbance spectrum of the trinitrophenyl moiety attached to the reactive lysyl residue of S-1 changed considerably upon addition of ATP or its analogues. ATP or AMP-PNP induced a larger spectral change than that of ADP, and its value was independent of the presence of Mg^{2+}. The trinitrophenylation of the reactive lysine considerably decreased the enhancement of the tryptophan fluorescence induced by MgATP, while it did not affect fluorescence enhancement induced by MgADP or MgAMP-PNP. The rate of formation of nucleotide-induced absorbance changes in trinitrophenylated (TNP) S-1 was followed in a stopped-flow spectrophotometer. The reaction could be described by a single exponential at every nucleotide concentration. The calculated apparent first-order rate constant, k_{obsd}, increased with rising concentrations of MgATP, ATP, MgADP, and MgAMP-PNP and finally reached a plateau. The absorbance change that appeared upon addition of MgATP to TNP S-1 eventually decayed in two phases until it reached the level of that induced by MgADP. The nucleotide concentration dependence of k_{obsd} deviated from a hyperbolic function in all cases studied. The nucleotide concentration dependence of the signal amplitude was also measured, and it was found to be independent of the presence of Mg^{2+}. The obtained results deviated from a computer-simulated binding curve, which was computed by assuming one identical binding site for each TNP S-1 molecule. The results indicate that an enzyme–substrate complex is the predominant intermediate in the TNP S-1 catalyzed MgATP hydrolysis, instead of an enzyme–product complex, which is the case with normal, unmodified S-1.

Myosin subfragment 1 (S-1)[1] is the segment of the myosin molecule responsible for ATPase activity and interaction with actin. A major effort was made in recent years to describe the molecular mechanism of ATP hydrolysis catalyzed by S-1, as well as the localized conformational changes coupled with the reaction. Several results pointed to localized conformational changes that are induced in myosin on addition of ATP or its analogues and on hydrolysis of the substrate. Changes were detected in the fluorescence intensity of 8-anilino-1-naphthalenesulfonate (ANS) bound to myosin (Cheung, 1969), in the environment of tryptophanyl residues, as indicated by ultraviolet (Morita, 1967) and fluorescence difference spectra (Werber et al., 1972) and in the ESR spectrum of spin-labels attached to the SH_1 thiol (Seidel et al., 1970). The conformation of the junction between the 27K and 50K fragments of S-1's heavy chain was also affected by the presence of nucleotides, as could be inferred from limited tryptic hydrolysis of S-1 (Muhlrad & Hozumi, 1982). The enhancement of tryptophan fluorescence, which was found to be associated with the binding of nucleotides as well as with the intermediate steps of hydrolysis of ATP, was used as a tool to elucidate the mechanism of this myosin-catalyzed process (Bagshaw & Trentham, 1974). However, in order to understand better this process, it would be desirable to study the kinetics of other conformational changes, which take place in well-defined locations in the S-1 segment and are coupled with ATP binding and hydrolysis.

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[1] Abbreviations: S-1, myosin subfragment 1; TNP S-1, trinitrophenylated S-1; RLR, reactive lysine residue in S-1; TNBS, 2,4,6-trinitrobenzenesulfonate; AMP-PNP, adenosine 5′-(β,γ-imidotriphosphate); Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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