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Evidence for similar conformational changes in α_2-macroglobulin on reaction with primary amines or proteolytic enzymes

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Reactions of α_2-macroglobulin (α_2M) with primary amines (ammonium chloride, methylammonium chloride and ethylammonium chloride) or proteolytic enzymes (trypsin, chymotrypsin and thrombin) resulted in changes of the absorption, fluorescence and circular-dichroism spectra and of the sedimentation coefficient of the inhibitor. All physico-chemical changes caused by the inactivation of α_2M by the amines were identical with, or highly similar to, those induced by the formation of the enzyme–inhibitor complexes. This suggests that similar conformational changes of the inhibitor occur in the two types of reactions. The frictional ratio, calculated from the increase in sedimentation coefficient, decreased from 1.67 for untreated α_2M to 1.57 for the amine- or proteinase-treated inhibitor. This change is due to a decrease in either asymmetry or hydration of the protein, resulting in a slightly smaller hydrodynamic volume. The circular-dichroism analyses indicated that the reaction of α_2M with either amines or proteinases is accompanied by a loss of the small amount (about 5%) of α-helix of the untreated protein. The changes of u.v. absorption and fluorescence suggested that about one out of the eight to ten tryptophan residues of each α_2M subunit is buried as a result of the conformational change. All spectroscopic and hydrodynamic changes that were observed are compatible with a spatial rearrangement of the subunits of α_2M, as implicated by the 'trap' hypothesis for the mechanism of inhibition of proteinases. However, a conformational change involving a decrease in the hydrodynamic volume of each subunit cannot be excluded.

α_2-Macroglobulin (α_2M) is a high-relative-molecular-mass ($M_r \sim 725000$), tetrameric plasma glycoprotein (Jones *et al.*, 1972; Hall & Roberts, 1978) that inhibits a wide variety of proteinases with different specificities (Barrett & Starkey, 1973; Harpel, 1976). It is generally acknowledged that each α_2M molecule can bind two molecules of proteinase (Ganrot, 1966; Barrett *et al.*, 1979; Swenson & Howard, 1979*a*; Sottrup-Jensen *et al.*, 1980; Pochon *et al.*, 1981). The binding is initiated by the proteinase cleaving the inhibitor in a limited region of the polypeptide chain, the 'bait' region (Harpel, 1973; Barrett *et al.*, 1979; Swenson & Howard, 1979*a*; Sottrup-Jensen *et al.*, 1981*b*). This cleavage triggers a non-covalent trapping of the enzyme, which greatly decreases the activity of the latter against high-M_r substrates (Barrett & Starkey, 1973; Harpel, 1976). Evidence from electron microscopy and gradient-gel electrophoresis indicates that the trapping of the proteinase is associated with a conformational change of the inhibitor (Barrett *et al.*, 1974, 1979). This change apparently also exposes one or more recognition sites on the inhibitor for the uptake and destruction of the inhibitor–proteinase complex by macrophages (Ohlsson, 1971; Debanne *et al.*, 1975; Kaplan & Nielsen, 1979*a*,*b*; Van Leuven *et al.*, 1979; Imber & Pizzo, 1981). Another sequel to the proteolytic cleavage is the hydrolysis of internal thioester bonds in the inhibitor, one in each polypeptide chain (Sottrup-Jensen *et al.*, 1980, 1981*a*; Howard, 1981; Salvesen *et al.*, 1981). This hydrolysis liberates a free thiol group (Sottrup-Jensen *et al.*, 1980) and an activated glutamic acid residue that can react covalently with amino groups on the proteinase

Abbreviations used: α_2M, α_2-macroglobulin; SDS, sodium dodecyl sulphate; IgM and IgA, immunoglobulins M and A; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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(Swenson & Howard, 1979*b*; Salvesen & Barrett, 1980; Salvesen *et al.*, 1981; Sottrup-Jensen *et al.*, 1981*c*; Wu *et al.*, 1981; Wang *et al.*, 1981; Van Leuven *et al.*, 1981*b*).

Certain primary amines are known to inactivate α_2M (Steinbuch *et al.*, 1968; Harpel, 1976; Barrett *et al.*, 1979). The inactivation is due to nucleophilic cleavage by the amine of the thioester bonds of the inhibitor, with the concurrent formation of an amide with the glutamic acid residue of the cleaved thioester (Swenson & Howard, 1979*b*, 1980; Sottrup-Jensen *et al.*, 1980; Howard, 1981). Studies by gradient-gel electrophoresis have indicated that the loss of activity is accompanied by a conformational change of the inhibitor that may be similar to that induced by the binding of proteinases (Barrett *et al.*, 1979; Van Leuven *et al.*, 1981*a*). A further indication of this similarity is that the macrophage recognition sites are exposed also on amine-inactivated α_2M (Kaplan *et al.*, 1981; Van Leuven *et al.*, 1981*a*; Imber & Pizzo, 1981).

Previous work has thus established that detailed information on the conformational changes of α_2M induced by amines or proteinases is essential for an understanding of the function of the inhibitor in the neutralization and elimination of potentially harmful enzymes. However, to date these conformational changes have only been cursorily studied. Here we therefore present a comprehensive characterization of these changes by spectroscopic and hydrodynamic methods.

Materials and methods

α_2M was prepared from fresh-frozen human plasma by precipitation with poly(ethylene glycol) (Barrett *et al.*, 1979), zinc-chelate chromatography (Kurecki *et al.*, 1979; Sottrup-Jensen *et al.*, 1980) and gel chromatography on Sephadex S-300 (Pharmacia, Uppsala, Sweden) in 50mM-sodium citrate/0.1M-NaCl, pH6.5 (Sottrup-Jensen *et al.*, 1980). The purified protein gave only one band on 4–30% (w/v)-polyacrylamide-gradient-gel electrophoresis (Barrett *et al.*, 1979), in immunoelectrophoresis against anti-human serum and in SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) on 5% (w/v) gels after treatment of the samples in 1% (w/v) SDS/0.3M-β-mercaptoethanol for 45min at 37°C. Single-radial-immunodiffusion analyses (Mancini *et al.*, 1965) showed the presence of less than 0.3% of either IgM, IgA, fibrinogen or haptoglobin. The preparation protected 1.7 ± 0.1 (S.D., $n = 6$) mol of trypsin/mol of α_2M from inhibition by soya-bean trypsin inhibitor (Barrett & Starkey, 1973; Sottrup-Jensen *et al.*, 1979). The purified protein was stored in the citrate buffer, pH6.5, at −70°C (Harpel, 1976) for up to 6 weeks. Gradient-gel electrophoresis (Barrett *et al.*, 1979) showed that no inactivation of the protein had occurred during this time. However, longer storage produced small amounts of aggregated material.

Bovine trypsin (type III, twice-crystallized; EC 3.4.21.4) and soya-bean trypsin inhibitor (type IS) were obtained from Sigma Chemical Co. β-Trypsin was isolated from the commercial preparation by affinity chromatography on agarose-linked soya-bean trypsin inhibitor (Robinson *et al.*, 1971; Yung & Trowbridge, 1975). Active-site titration with 4-nitrophenyl 4-guanidinobenzoate (Chase & Shaw, 1970), obtained from E. Merck, Darmstadt, Germany, gave 0.87 ± 0.03 (S.D., $n = 9$) mol of active sites/mol of protein. α-Chymotrypsin A (thrice-crystallized; EC 3.4.21.1) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. The preparation had 1.04 ± 0.04 (S.D., $n = 9$) and 0.90 ± 0.05 (S.D., $n = 7$) mol of active sites/mol of protein by active-site titrations with 4-nitrophenyl acetate (Serva, Heidelberg, Germany) and 4-nitrophenyltrimethyl acetate (EGA-Chemie, Steinheim, Germany; recrystallized three times before use) respectively (Kézdy & Kaiser, 1970). A value of 0.95mol of active sites/mol protein was used throughout the work. Bovine α-thrombin (EC 3.4.21.5) was isolated after activation of purified prothrombin, as described previously (Carlström *et al.*, 1977). Active-site titration with 4-nitrophenyl 4-guanidinobenzoate gave 0.85 ± 0.02 (S.D., $n = 4$) mol of active sites/mol of protein. The solvent for trypsin and chymotrypsin was 1mM-HCl/10mM-$CaCl_2$, whereas thrombin was dissolved in 50mM-Hepes/0.1M-NaCl, pH8.2.

U.v.-absorption difference spectra were measured in a Cary model 219 spectrophotometer (Varian Instruments, Palo Alto, CA, U.S.A.). The measurements were done at 25.0 ± 0.2°C with tandem cells having 1cm path-lengths per compartment and with α_2M concentrations of 1.9–2.3μM (i.e. about 1.4–1.7g/litre). The two compartments of each tandem cell initially contained 2.00ml of either α_2M solution or buffer. Identical volumes (50–200μl) of amine or enzyme solution were added to the protein compartment of the sample cell and to the buffer compartment of the reference cell. The same volume of buffer was also added to the protein compartment of the reference cell. The absorption difference spectrum between sample and reference was then measured. The results were expressed as differences in molar absorption between sample and reference.

Fluorescence was measured at 25.0 ± 0.2°C in an Aminco SPF 500 spectrofluorimeter (American Instrument Company, Silver Spring, MD, U.S.A.). Corrected emission spectra were recorded with excitation and emission bandwidths of 2nm; the excitation wavelength was 280nm. Concentrations of α_2M were about 275 and 70nM (i.e. about 200

and 50mg/litre) in experiments with amines and proteinases respectively. Separate measurements were made of sample containing α_2M and amine or enzyme, a reference solution of α_2M with the same concentration as in the sample, a solution of amine or enzyme alone, also with the same concentration as in the sample, and buffer. The fluorescence of the amines was negligible, but that of the free enzymes (corrected for the inner-filter effect due to the α_2M in the sample) was subtracted from the sample fluorescence. All other corrections for inner-filter effects were insignificant at the protein concentrations used.

Circular dichroism was measured at room temperature ($22 \pm 2°C$) with a Jasco J 41 A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). Measurements in the far-u.v.(200–250nm) wavelength region were done with cells with 0.1cm path-lengths and with α_2M concentrations of about 480nM (i.e. about 0.35g/litre), whereas cells with 1cm path-lengths and α_2M concentrations of about 2.1μM (i.e. about 1.5g/litre) were used in the near-u.v. (250–310nm) region. For each sample, a solution of amine or proteinase of the same concentration as in the sample was used as a blank. The results were expressed as mean-residue ellipticities in the far-u.v. region and as molar ellipticities in the near-u.v. region. In the calculations of mean-residue ellipticities, the concentration (assuming 8% carbohydrate) and the mean residue weight (111) of the peptide moiety of α_2M were used (Hall & Roberts, 1978).

Sedimentation-velocity analyses were done in a Beckman model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.). α_2M (3–8g/litre) was first treated with amine or proteinase, which was then removed by gel chromatography on Sephadex G-25 or CL-Sepharose 6B (Pharmacia) respectively. All ultracentrifuge measurements were done within 10h after inactivation was started. Some samples were measured again 24h after their preparation and showed no change in sedimentation rate. The analyses were carried out as differential sedimentation-velocity analyses, essentially as described by Schumaker & Adams (1968), except that the scanning absorption optical system was used in nearly all experiments. The measurements were made at 44000rev./min and 20.8°C. Since the cells could not be scanned simultaneously, the published procedure had to be modified as follows. Three solutions were run in each experiment: untreated α_2M as a reference at a constant concentration of 2.0g/litre, and samples of α_2M, untreated or treated with amine or enzyme, at concentrations which varied between 2 and 0.3g/litre. Each cell was scanned at least seven times during a run, and the data for each cell was fitted by a linear least-squares procedure to eqn. (3) of Schumaker & Adams (1968). The difference in sedimentation coefficient between each experimental sample and the α_2M reference was then estimated from the least-squares fits, essentially as in the original procedure (Schumaker & Adams, 1968).

Most spectroscopic and all sedimentation-velocity analyses, including preparations of samples for the latter, were done in 50mM-Hepes/0.1M-NaCl, pH8.2. However, owing to the far-u.v. absorbance of this buffer, 50mM-Tris/HCl/0.1M-NaCl, pH8.2, was used in the circular-dichroism analyses in this wavelength region. Prolonged exposure (>2 days) of α_2M to the Tris buffer was found to result in the appearance of small amounts of the inactive form of the protein (Barrett *et al.*, 1979). Experiments with α_2M in the Tris buffer were therefore started immediately after transfer of the sample into this buffer by gel chromatography on Sephadex G-25.

Protein concentrations were obtained by absorption measurements at 280nm. Specific absorption coefficients (in litres$\cdot g^{-1}\cdot cm^{-1}$) of 0.90 for α_2M (Dunn & Spiro, 1967; Hall & Roberts, 1978), 1.54 for trypsin (Robinson *et al.*, 1971), 2.05 for chymotrypsin (Wilcox, 1970) and 1.75 for thrombin (Fish *et al.*, 1979) were used. Relative molecular masses of 725000 for α_2M (Jones *et al.*, 1972; Hall & Roberts, 1978), 23300 for trypsin (Walsh & Neurath, 1964), 25100 for chymotrypsin (Hartley, 1964; Hartley & Kauffman, 1966) and 37000 for thrombin (Magnusson *et al.*, 1975) were used for calculating molar concentrations.

Results

Spectroscopic changes

Preliminary kinetic analyses showed that, under the conditions chosen, i.e. 100mM-amine, pH8.2, 25°C, the spectroscopic changes observed in the reactions of α_2M with two of the amines used, ammonium chloride and methylammonium chloride, were more than 95% complete after 120min. All spectroscopic analyses with these two amines were thus done after a reaction time of 120–150min. In contrast, the reaction with the third amine, ethylammonium chloride, was appreciably slower under the same conditions and could be monitored to similar near-completion, i.e. for 240min, only in the fluorescence analyses.

The spectral changes appearing in the reactions between α_2M and the three proteinases studied, trypsin, chymotrypsin and thrombin, all with known contents of active sites, developed rapidly, i.e. in less than 2–5min; all measurements were made on samples that were allowed to react for 5–15min. The analyses were done with an active-proteinase/inhibitor molar ratio of 2.5–3.0. Control experiments showed that higher ratios caused no further spectroscopic changes, but only led to larger experimental

error due to the contributions by the enzymes to the observed spectra.

U.v. difference spectroscopy. Difference spectra between α_2M allowed to react with amines and the untreated protein were measured in the near-u.v. wavelength region (Fig. 1*a*). The spectra produced by ammonium chloride and methylammonium chloride are clearly identical within the errors of the measurements, which are rather high owing to the low magnitude of the spectra. The features of these two spectra are also evident in the spectrum given by ethylammonium chloride, but the magnitude of this spectrum is lower, since the reaction was not complete when the measurements were made, i.e. after 120 min. Measurements after longer reaction times gave considerably increased error, owing to baseline drift and development of a small amount of turbidity. The overall shape of the spectra is that expected from a red shift accompanied by an increase in absorption. However, the spectra show considerable additional detail, with distinct maxima at 295, 290, 282 and 275 nm, and several shoulders below 270 nm.

The difference spectra measured between the α_2M–proteinase complexes and the inhibitor alone are also similar to each other, although their magnitudes differ somewhat (Fig. 1*b*). They are also highly similar to, but not identical with, the spectra given by the amines. The main difference is that the positive band at 295 nm in the spectra due to the amines is not seen in the spectra caused by the proteinases.

Fluorescence. The corrected fluorescence emission spectrum obtained for α_2M alone is a typical tryptophan emission spectrum with a maximum at 326 nm (Fig. 2*a*). Reaction with amines caused about a 3 nm blue shift of the emission maximum and about a 45% enhancement of the fluorescence. No differences among the three amines were observed, since with fluorescence the reaction with ethylammonium chloride could also be followed to near-completion.

Analyses of the fluorescence changes caused by the enzymes were done at a protein concentration four times lower than that used in the amine experiments. This slightly increased experimental

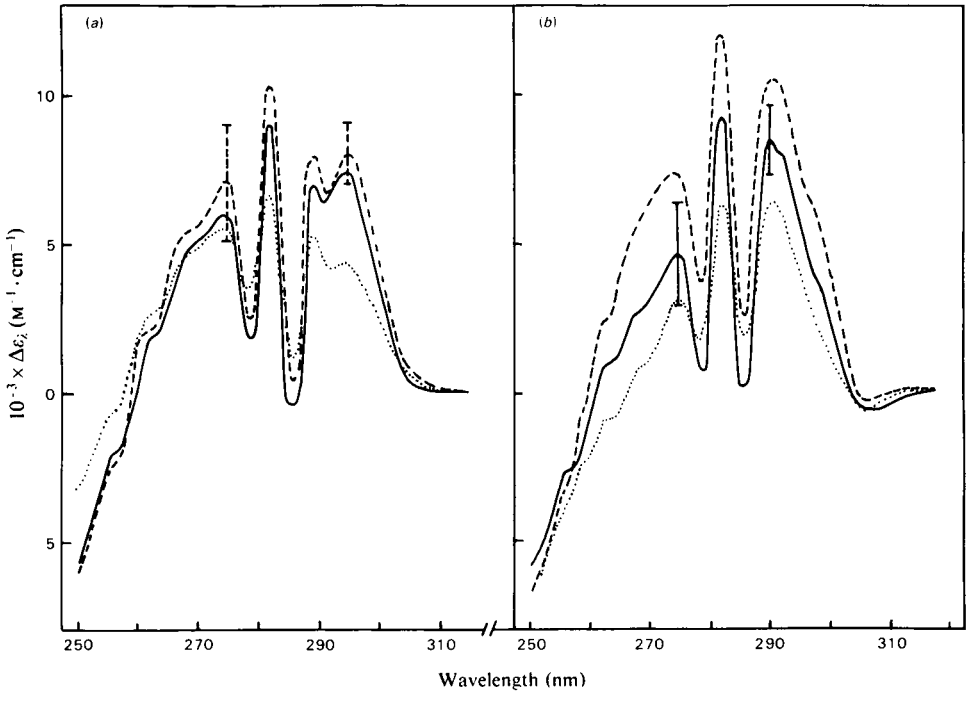

Fig. 1. *U.v. absorption difference spectra measured between α_2M treated with amines or proteinases and α_2M alone*
(*a*) α_2M allowed to react with amines: ——, ammonium chloride; – – – –, methylammonium chloride: • • • •, ethylammonium chloride. (*b*) α_2M allowed to react with proteinases: ——, trypsin; – – – –, chymotrypsin; • • • •, thrombin. The error bars represent S.D. calculated from three separate analyses; curves without S.D. bars show average results from two analyses.

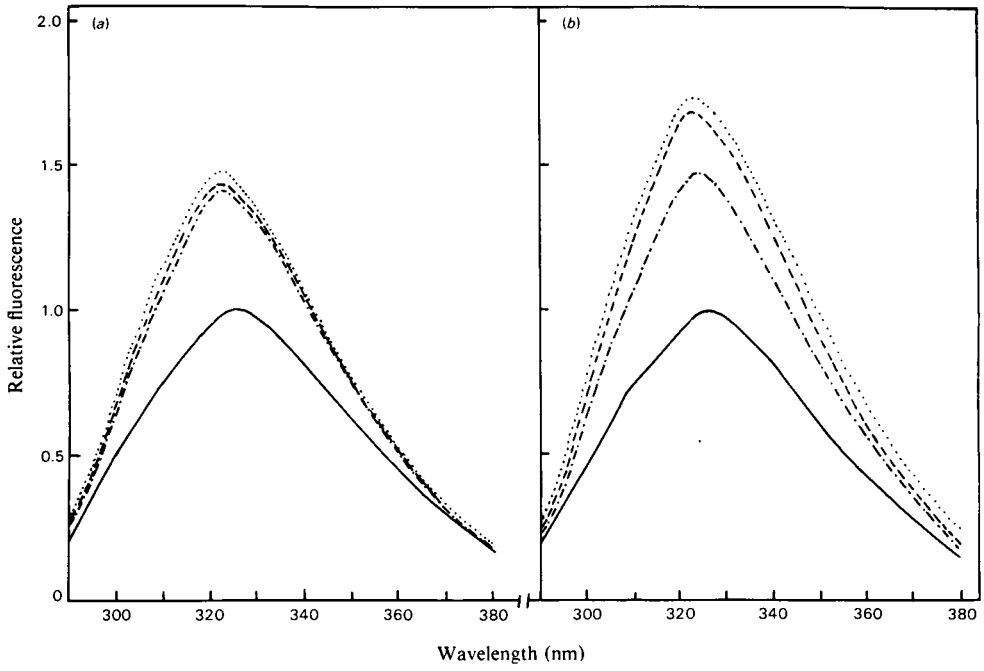

Fig. 2. *Corrected fluorescence spectra of α_2M alone and α_2M allowed to react with amines or proteinases*
——, α_2M alone. (*a*) α_2M allowed to react with amines: – – –, ammonium chloride; ····, methylammonium chloride; – · –, ethylammonium chloride. (*b*) α_2M allowed to react with proteinases: – – –, trypsin; ····, chymotrypsin; – · –, thrombin.

error, but was necessary to minimize the inner-filter effects in these analyses. All three enzymes produced the same blue shift as the amines, but two of them, trypsin and chymotrypsin, gave a slightly higher fluorescence enhancement, about 70% (Fig. 2*b*). An appreciably lower fluorescence enhancement, about 30%, has been observed for trypsin in a previous investigation (Richman & Verpoorte, 1981).

Circular dichroism. Circular-dichroism spectra were measured both in the far-u.v. and in the near-u.v. wavelength regions. Only the effects of two amines, ammonium chloride and methylammonium chloride, and two proteinases, trypsin and chymotrypsin, were studied. The far-u.v. circular-dichroism spectrum of α_2M alone shows a minimum at about 214nm and a shoulder around 210nm (Fig. 3). Reaction of the inhibitor with either amines or enzymes caused a small but reproducible increase in ellipticity and a loss of the 210nm shoulder; these changes were identical within experimental error for the two types of reagents. The spectrum of α_2M alone and the ellipticity change caused by the reaction with trypsin are similar to the results reported in a recent paper (Richman & Verpoorte, 1981). However, the spectrum of the unchanged inhibitor differs markedly from that obtained in a previous investigation (Frénoy *et al.*, 1977). The latter is more like the spectrum measured for the inactivated protein in the present work, although the ellipticity is even higher. Since the preparation of the α_2M studied by Frénoy *et al.* (1977) involved precipitation with $(NH_4)_2SO_4$, it is possible that considerable inactivation of the inhibitor occurred during the isolation procedure.

The aromatic circular-dichroism spectrum measured for α_2M (Fig. 4) indicates contributions from tryptophan, tyrosine and possibly also phenylalanine residues. Reaction with amines caused moderate, but reproducible, changes of this spectrum, which were similar for the two amines (Fig. 4*a*). The changes produced by the enzymes were smaller and experimentally less certain; however, the changes that were observed occurred in the same wavelength regions as some of the amine-induced changes.

Hydrodynamic changes

For the sedimentation-velocity analyses, α_2M was allowed to react with amines (ammonium chloride and methylammonium chloride only) as in the spectroscopic analyses, i.e. with 100mM-amine for 120min at 25°C. The reactions with the proteinases

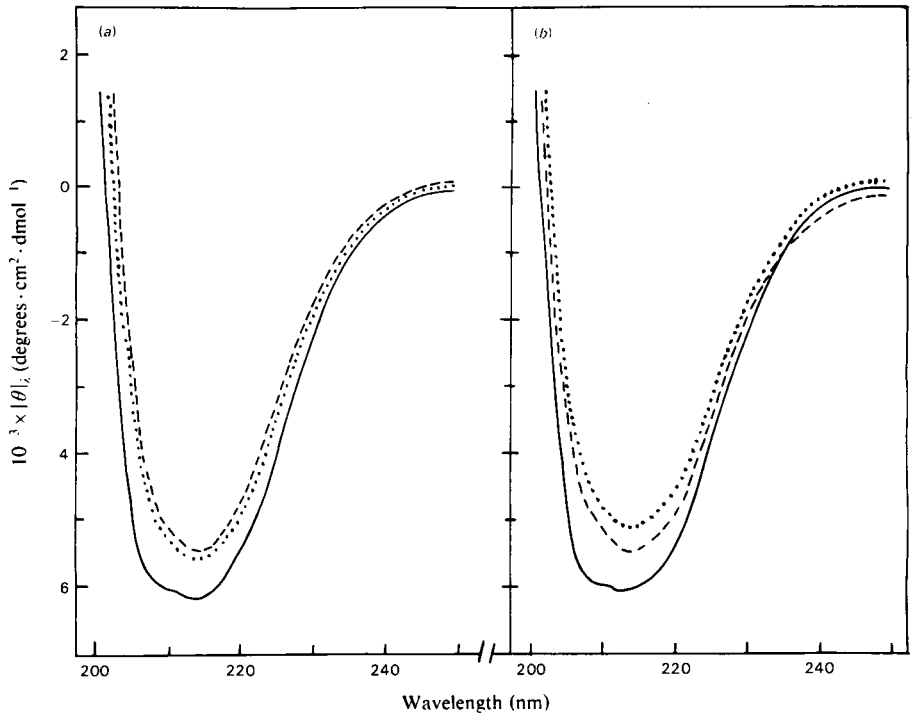

Fig. 3. *Far-u.v. circular-dichroism spectra of* α_2*M alone and* α_2*M allowed to react with amines or proteinases* ——, α_2M alone. (*a*) α_2M allowed to react with amines: – – – –, ammonium chloride; ····, methylammonium chloride. (*b*) α_2M allowed to react with proteinases: – – – –, trypsin; ····, chymotrypsin.

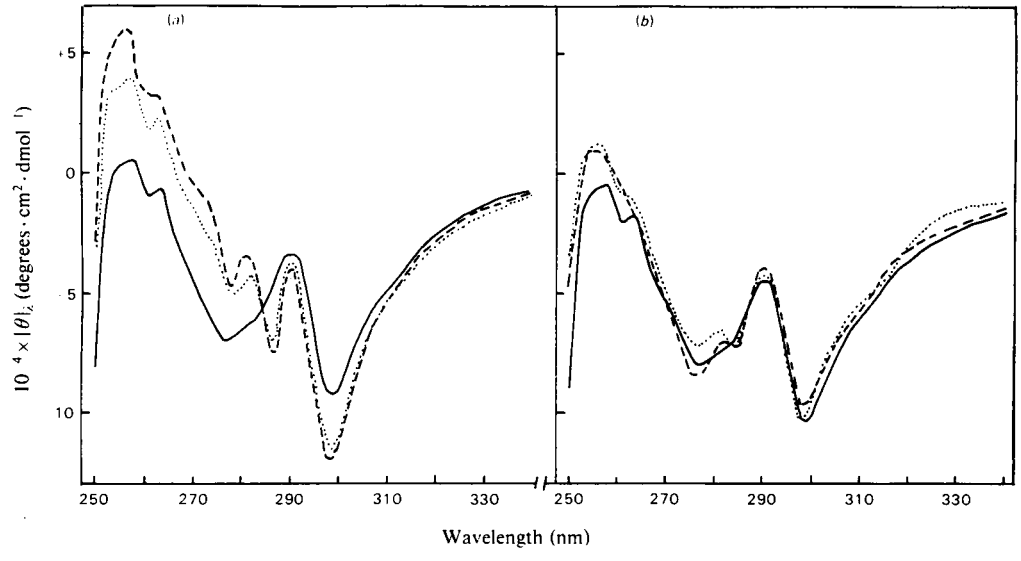

Fig. 4. *Near-u.v. circular-dichroism spectra of* α_2*M alone and* α_2*M allowed to react with amines or proteinases* ——, α_2M alone. (*a*) α_2M allowed to react with amines: – – – –, ammonium chloride; ····, methylammonium chloride. (*b*) α_2M allowed to react with proteinases: – – – –, trypsin; ····, chymotrypsin.

(trypsin and chymotrypsin only) were done at the same pH and temperature for 15 min at an active-enzyme/inhibitor molar ratio of 2.2. In both cases, excess reagent was removed by gel chromatography before analyses. The amount of proteinase was chosen to minimize the binding of enzyme that occurs at higher ratios of proteinase to inhibitor by a mechanism not involving 'trapping' of the enzyme and that thus may be non-specific (Sottrup-Jensen *et al.*, 1981*d*). This additionally bound proteinase can be dissociated from α_2M by low-relative-molecular-mass protein proteinase inhibitors (Sottrup-Jensen *et al.*, 1981*d*). Thus, in some experiments with trypsin, soya-bean trypsin inhibitor was incubated before gel chromatography with the α_2M/trypsin reaction mixture for 15 min at a molar ratio to the enzyme of 2.0. As a control to these experiments, trypsin and soya-bean trypsin inhibitor were first allowed to react for 1 h at the same molar ratio, and this mixture was then added 15 min before gel chromatography to α_2M in an amount corresponding to an enzyme/α_2M molar ratio of 2.2.

The changes of the sedimentation coefficient of α_2M accompanying the reactions were characterized by differential sedimentation-velocity analyses, since only small effects were anticipated. Although most analyses were done with the scanning absorption optical system, identical sedimentation coefficients were obtained with schlieren optics at the highest sample concentrations. All analyses gave sedimentation boundaries indicating more-than-95% size homogeneity of the samples. The observed difference in sedimentation coefficient between the sample and a reference solution of untreated α_2M was plotted against the protein concentration of the sample (Fig. 5). All data for each of the three forms of α_2M, i.e. untreated, amine-inactivated or proteinase-treated, were subjected to common least-squares regression analyses, since no differences within the three data sets were apparent. The small positive slope of the resulting regression lines (Fig. 5) may indicate a slight tendency for both active and inactivated α_2M to aggregate reversibly at increasing protein concentrations. Sedimentation coefficients at infinite dilution for the three forms of α_2M were calculated from the sedimentation-coefficient differences, extrapolated to zero protein concentration, and the accurately determined sedimentation coefficient of the α_2M reference (Table 1). Sedimentation coefficients were also calculated in the conventional manner from the same experiments. These values showed no or a slight positive dependence on protein concentration; however, the plots are not presented. Extrapolation to zero protein concentration gave $s^0_{20,w}$ values that agree well with those determined by the difference procedure (Table 1).

Frictional ratios were calculated from the sedimentation coefficients at infinite dilution (Table 1). The partial specific volume of untreated α_2M, 0.731 ml/g (Hall & Roberts, 1978) was also used in

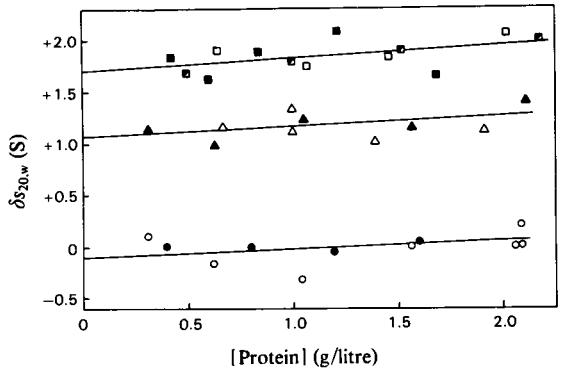

Fig. 5. *Sedimentation coefficient differences, $\delta s_{20,w}$, between untreated α_2M, amine-inactivated α_2M or α_2M–proteinase complexes at various concentrations and a reference solution of untreated α_2M at 2.0 g/litre*

○, untreated α_2M; ●, untreated α_2M incubated with a soya-bean trypsin inhibitor–trypsin mixture; △, α_2M allowed to react with ammonium chloride; ▲, α_2M allowed to react with methylammonium chloride; □, α_2M allowed to react with trypsin; ■, α_2M allowed to react with chymotrypsin; ◪, α_2M allowed to react with trypsin and then treated with soya-bean trypsin inhibitor.

Table 1. *Sedimentation coefficients and frictional ratios (f/f_{min}) of untreated α_2M, amine-inactivated α_2M and α_2M–proteinase complexes*

α_2M	Difference in $s^0_{20,w}$ between sample and reference* (S)	$s^0_{20,w}$ from differential sedimentation† (S)	$s^0_{20,w}$ from direct plot (S)	$s^0_{20,w}$ used for f/f_{min} (S)	f/f_{min}
Untreated	−0.11 ± 0.15	17.2	17.3	17.3	1.67
Amine-inactivated	+1.07 ± 0.13	18.4	18.4	18.4	1.57
As a complex with proteinase	+1.69 ± 0.13	19.0	19.3	19.2	1.57

* The reference was untreated α_2M at 2.0 g/litre; the errors are the 95% confidence intervals around the extrapolated values.

† Calculated from an $s_{20,w}$ of 17.34 ± 0.14 S (S.D., $n = 20$) for the α_2M reference.

these calculations for the amine- or proteinase-treated inhibitor. This assumption is reasonable, since the partial specific volume of both trypsin and chymotrypsin is close to 0.73 ml/g (Tietze, 1953; Schwert & Kaufman, 1951) and the enzymes constitute only a small weight fraction of the complexes. The M_r of the α_2M–protease complexes was taken as the sum of the M_r of the inhibitor alone and twice the average M_r of the enzymes. Both amines and proteinases were found to cause the same decrease of the frictional ratio of α_2M. The higher sedimentation coefficient of the complexes between the inhibitor and proteinases compared with that of the amine-inactivated protein (Table 1) thus reflects primarily the greater mass of these complexes.

The sedimentation coefficient at infinite dilution of active α_2M obtained in the present work, 17.3 S, is slightly lower than the values of 18.1 and 18.7 S reported previously (Jones *et al.*, 1972; Hall & Roberts, 1978). Part of this discrepancy may be due to the presence of inactivated protein in the preparations used in the previous studies. Also, higher protein concentrations were used in these investigations, and therefore longer and less certain extrapolations to zero protein concentration were necessary.

Discussion

The results presented suggest that the spectroscopic and hydrodynamic changes observed in the reactions of α_2M both with amines and with proteinases all reflect altered conformations of the inhibitor. The changes in frictional ratio and far-u.v. circular dichroism thus strongly indicate changes of both the shape and secondary structure of α_2M. Moreover, the possibility that the near-u.v. spectral changes primarily are due to only local interactions between α_2M and the bound amine or proteinase without a conformational change is highly unlikely. Such local interactions thus would not be expected to give rise to the identical or very similar spectroscopic changes observed for the different amines or proteinases with each of the three methods used. The similarity of the active sites of the three serine proteinases does not contradict this conclusion, since the active sites are not directly involved in the interactions with the inhibitor. The additional possibility, that the near-u.v. spectroscopic changes observed in the reactions with the proteinases reflect conformational changes of the latter instead of the inhibitor, is also improbable, since similar changes were obtained for three different enzymes with different three-dimensional structures, and also since the spectral changes due to the enzymes were similar to those caused by the amines.

A further conclusion of the present work is that the conformational change of α_2M caused by primary amines is highly similar to that caused by proteinases. The frictional ratios and far-u.v. circular-dichroism spectra of the inhibitor measured after the reactions thus were identical for the two types of reagents. Moreover, only small differences in the aromatic wavelength region were observed with the other spectroscopic techniques. This indicates only minor differences in local conformation around aromatic residues between the two α_2M forms that had reacted. These differences may be caused by the scission of the polypeptide chain of the inhibitor, which occurs only in the reaction with proteinases. Minor contributions to the proteinase-induced spectra from local interactions between enzyme and inhibitor are a further possibility; such interactions may also be responsible for the small differences between the spectroscopic changes caused by the three proteinases.

The results also allow several conclusions on the nature of the conformational change of α_2M common to the reactions with amines or proteinases. The high frictional ratio of the untreated inhibitor may be due to an asymmetric shape or arrangement of the subunits of the protein or to trapping of a considerable amount of solvent between the subunits. The decrease in frictional ratio on reaction with amines or proteinases must be caused by a decrease in either asymmetry or hydration, or in both, resulting in a slightly smaller hydrodynamic volume of the inhibitor. Such a change is in agreement with earlier studies by electron microscopy and polyacrylamide-gel electrophoresis (Barrett *et al.*, 1974, 1979). However, the hydrodynamic change apparently is sufficiently small not to have been detected by the triplet-probe-depolarization technique (Pochon *et al.*, 1978).

The far-u.v. circular-dichroism spectra suggest that the change of the hydrodynamic properties of α_2M is accompanied by a slight decrease of the α-helical content of the inhibitor. Evaluation of these spectra by the methods described by Greenfield & Fasman (1969) and Chen *et al.* (1972) thus indicate that untreated α_2M has a small amount, about 5%, of α-helix and 35–60% of β-structure, whereas the amine- or proteinase-treated inhibitor has no α-helix and 40–65% β-structure. However, in spite of the fact that the analyses by the two procedures agree, interpretation of such a small circular-dichroism change in absolute structural terms is uncertain.

The maxima at 290–295 nm and 282 nm in the absorption difference spectra and the fluorescence enhancement with a maximum at about 323 nm show that the near-u.v. spectroscopic changes associated with the conformational change of α_2M are due primarily to perturbation of tryptophan

residues (Chen *et al.*, 1969; Donovan, 1973). Moreover, both the absorption red shift and the fluorescence blue shift indicate that the tryptophan residues are transferred to a more hydrophobic environment (Chen *et al.*, 1969; Donovan, 1973). Comparison of the observed absorption change with that estimated for the transfer of one tryptophan residue from the surface to the interior of the protein ($\Delta\varepsilon_{292} \sim 1600\,M^{-1}\cdot cm^{-1}$; Donovan, 1964, 1973) suggests that four to five residues per α_2M molecule, i.e. about one of the eight to ten tryptophan residues of each subunit (Hall & Roberts, 1978), are buried. This number may represent the complete burial of one particular tryptophan residue or only the partial burial of several residues.

In summary, all physico-chemical changes observed in the present work are compatible with a spatial rearrangement of the subunits of α_2M after reaction with amines or proteinases (Barrett *et al.*, 1979). However, other interpretations, such as a change of conformation involving a decrease in the hydrodynamic volume of each subunit, are also possible.

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