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The conformational changes of α_2-macroglobulin induced by methylamine or trypsin

Characterization by extrinsic and intrinsic spectroscopic probes

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The conformational changes around the thioester-bond region of human or bovine α_2M (α_2-macroglobulin) on reaction with methylamine or trypsin were studied with the probe AEDANS [*N*-(acetylaminoethyl)-8-naphthylamine-1-sulphonic acid], bound to the liberated thiol groups. The binding affected the fluorescence emission and lifetime of the probe in a manner indicating that the thioester-bond region is partially buried in all forms of the inhibitor. In human α_2M these effects were greater for the trypsin-treated than for the methylamine-treated inhibitor, which both have undergone similar, major, conformational changes. This difference may thus be due to a close proximity of the thioester region to the bound proteinase. Reaction of trypsin with thiol-labelled methylamine-treated bovine α_2M, which retains a near-native conformation and inhibitory activity, indicated that the major conformational change accompanying the binding of proteinases involves transfer of the thioester-bond region to a more polar environment without increasing the exposure of this region at the surface of the protein. Labelling of the transglutaminase cross-linking site of human α_2M with dansylcadaverine [*N*-(5-aminopentyl)-5-dimethylaminonaphthalene-1-sulphonamide] suggested that this site is in moderately hydrophobic surroundings. Reaction of the labelled inhibitor with methylamine or trypsin produced fluorescence changes consistent with further burial of the cross-linking site. These changes were more pronounced for trypsin-treated than for methylamine-treated α_2M, presumably an effect of the cleavage of the adjacent 'bait' region. Solvent perturbation of the u.v. absorption and iodide quenching of the tryptophan fluorescence of human α_2M showed that one or two tryptophan residues in each α_2M monomer are buried on reaction with methylamine or trypsin, with no discernible change in the exposure of tyrosine residues. Together, these results indicate an extensive conformational change of α_2M on reaction with amines or proteinases and are consistent with several aspects of a recently proposed model of α_2M structure [Feldman, Gonias & Pizzo (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 5700–5704].

INTRODUCTION

α_2-Macroglobulin (α_2M) is a large ($M_r \sim 725000$) tetrameric plasma proteinase inhibitor that inhibits a wide variety of proteinases (Jones *et al.*, 1972; Barrett & Starkey, 1973; Harpel, 1976; Hall & Roberts, 1978). The binding of the enzyme is associated with a conformational change of the inhibitor, initiated by proteolytic cleavage of a specific region of the polypeptide chain, the 'bait' region (Harpel, 1973; Barrett & Starkey, 1973; Barrett *et al.*, 1979; Swenson & Howard, 1979; Sottrup-Jensen *et al.*, 1981*a*; Gonias *et al.*, 1982; Björk & Fish, 1982; Schramm & Schramm, 1982; Dangott *et al.*, 1983). This conformational change alters both the secondary and tertiary structure, as well as the shape, of α_2M. The proteinase is bound to α_2M in a manner that blocks its activity only against high-molecular-mass substrates and may thus be physically entrapped by the inhibitor (Barrett & Starkey, 1973). Reaction with the proteinase also leads to exposure of a receptor-recognition site involved in uptake of the α_2M–proteinase complex into cells (Van Leuven *et al.*, 1979). Each molecule of α_2M can bind maximally two molecules of enzyme, indicating 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; Pochon *et al.*, 1981; Gonias & Pizzo, 1983; Björk *et al.*, 1984).

A distinctive feature of the structure of α_2M is a thioester bond between a cysteine and a glutamic acid residue, separated by only two interjacent residues, in each polypeptide chain of the inhibitor (Sottrup-Jensen *et al.*, 1980; Howard, 1981; Salvesen *et al.*, 1981). This bond is cleaved on reaction with the proteinase, leading to liberation of a thiol group and to covalent linkage of some enzyme molecules to the inhibitor via the glutamic acid residue (Salvesen & Barrett, 1980; Sottrup-Jensen *et al.*, 1981*b*; Wu *et al.*, 1981). However, the covalent binding is not necessary for the inhibition. The thioester bonds can also be cleaved by a nucleophilic attack by small primary amines (Sottrup-Jensen *et al.*, 1980; Howard, 1981; Salvesen *et al.*, 1981; Larsson & Björk, 1984; Strickland & Bhattacharya, 1984). In human α_2M, this cleavage leads to a series of conformational changes, the final state being inactive and having a conformation

Abbreviations used: α_2M, α_2-macroglobulin; Ac-Cys, *N*-acetylcysteine; AEDANS, *N*-(acetylaminoethyl)-8-naphthylamine-1-sulphonic acid; I-AEDANS, *N*-(iodoacetylaminoethyl)-8-naphthylamine-1-sulphonic acid; dansylcadaverine, *N*-(5-aminopentyl)-5-dimethylaminonaphthalene-1-sulphonamide.

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similar to that induced by proteinases (Barrett *et al.*, 1979; Gonias *et al.*, 1982; Björk & Fish, 1982; Strickland & Bhattacharya, 1984; Larsson *et al.*, 1985; Eccleston & Howard, 1985). In contrast, no major conformational change occurs in bovine α_2M, and this protein also retains activity. However, the apparent stoichiometry and the rate of binding of proteinases is reduced, and the binding of certain fluorescent probes is altered, indicative of a limited change of conformation (Dangott & Cunningham, 1982; Strickland & Bhattacharya, 1984; Björk *et al.*, 1985). Nevertheless, reaction of methylamine-treated bovine α_2M with proteinases induces a similar conformational change in the modified as in the native inhibitor (Björk *et al.*, 1985).

The nature of the conformational changes in α_2M on reaction with proteinases or amines, and the relation of these changes to 'bait'-region and thioester-bond cleavage, remain largely unclear, although an attractive, but speculative, model has been presented (Feldman *et al.*, 1985). In an attempt to elucidate these aspects of α_2M function further, we have characterized the conformational changes occurring in the inhibitor on treatment with trypsin or methylamine by several spectroscopic techniques. The approach includes the use of fluorescent probes bound to the thiol group of the cleaved thioester bond and to the transglutaminase-reactive site of the protein, as well as analyses of the intrinsic spectroscopic properties of the aromatic amino acids of the protein.

MATERIALS AND METHODS

α_2M was isolated from human or bovine plasma and β-trypsin (EC 3.4.21.4) from commercial bovine trypsin (Type III; Sigma Chemical Co., St. Louis, MO, U.S.A.), as described previously (Björk & Fish, 1982; Björk *et al.*, 1985). Active-site titrations of the enzyme with 4-nitrophenyl 4-guanidinobenzoate (E. Merck, Darmstadt, Germany; Chase & Shaw, 1970), gave 0.89 ± 0.01 (S.D., $n = 9$) mol of active sites/mol of protein.

Bovine plasma transglutaminase zymogen (Factor XIII) was purified by published procedures (Lorand & Gotoh, 1970; Curtis & Lorand, 1976). The preparation had a specific activity of 500 amine incorporation units (Lorand & Gotoh, 1970) per mg of protein. The activity in 1 ml of human plasma, measured by the same assay, was about 25 units.

The thiol group liberated in human or bovine α_2M on reaction with methylamine or trypsin was labelled with I-AEDANS (Hudson & Weber, 1973) in the following manner. α_2M (final concn. ~ 2.5 μM) in 50 mM-Hepes/NaOH/0.1 M-NaCl/2 mM-EDTA, pH 8.0, was incubated at 25 °C for 2 h with 100 mM-methylamine (Merck) or for 10 min with an amount of trypsin just sufficient to saturate the inhibitor, i.e. at trypsin/α_2M molar ratios of 2.2 for the human and 1.7 for the bovine inhibitor (Björk *et al.*, 1984, 1985). The reaction mixtures also contained I-AEDANS (Serva, Heidelberg, Germany) at concentrations of 80 μM and 500 μM for human and bovine α_2M respectively. The reactions with trypsin were stopped by addition of *N*-α-*p*-tosyl-L-lysylchloromethane ('TLCK') (Sigma) to a concentration of 2 mM. Both methylamine- and trypsin-treated samples were immediately diluted 10-fold with Hepes buffer and reconcentrated to the original volume by ultrafiltration with an XM 300 membrane (Amicon Corp., Lexington, MA, U.S.A.). This cycle was repeated once, and the samples were then dialysed against 400 vol. of buffer overnight. Control experiments showed that this treatment reduced the concentrations of trypsin, which passes through the ultrafiltration membrane, and that of the fluorescent reagent to insignificant levels. The extent of labelling was estimated from the A_{342} of a solution passed through a filter with a pore size of 0.45 μm. The absorbance of a similarly filtered sample of untreated α_2M of the same concentration was subtracted from this value, and the concentration of bound fluorophore was calculated from the corrected absorbance with the use of a molar absorption coefficient of 6300 $M^{-1} \cdot cm^{-1}$ (Hudson & Weber, 1973). Ac-Cys-AEDANS was prepared by reaction of a known concentration of I-AEDANS with a 10-fold excess of Ac-Cys (Serva).

For the incorporation of dansylcadaverine into α_2M, plasma transglutaminase [100 μg in 225 μl of 50 mM-Tris/HCl/20 mM-GSH/120 mM-$CaCl_2$/15% (v/v) glycerol, pH 7.5] was activated with 5 μg of purified thrombin (Björk, 1985) for 30 min at 25 °C. The activated enzyme was then added to 3 ml of human α_2M (5.5 μM) in 50 mM-Hepes/NaOH/2 mM-dansylcadaverine (Fluka, Buchs, Switzerland)/1 mM $CaCl_2$, pH 7.5. After incubation for 1 h at 25 °C, another portion of activated enzyme was added, and the incubation was continued for an additional 4 h. The reagents were then removed by gel chromatography (10 ml/h) at 4 °C on a column (1.6 cm × 90 cm) of Sepharose 6B (Pharmacia, Uppsala, Sweden) in 50 mM-Tris/HCl/1 mM-EDTA, pH 7.5. The extent of incorporation of dansylcadaverine into α_2M was determined from the A_{340}, measured as described for the I-AEDANS-labelling procedure, with the use of a molar absorption coefficient of 3400 $M^{-1} \cdot cm^{-1}$ (Chen, 1968; Van Leuven *et al.*, 1981).

Corrected fluorescence emission spectra of the AEDANS and dansylcadaverine fluorophores were measured at 25.0 ± 0.1 °C in a SLM 4800S spectrofluorimeter (SLM–Aminco, Urbana, IL, U.S.A.). The excitation wavelength was 340–342 nm, and the bandwidths were 4–8 nm in both channels. Protein concentrations were 0.27–0.35 μM, giving fluorophore concentrations of 1.0–1.6 μM. The samples were continuously stirred during measurements. Quantum yields were calculated from the areas under the corrected emission spectra of solutions of sample and a standard [quinine bisulphate (Fluka) in 0.5 M-H_2SO_4] having the same absorbance at the excitation wavelength. A quantum yield of 0.55 was used for the standard (Bridges, 1981).

Fluorescence lifetimes were measured at 25.0 ± 0.1 °C with the phase-modulation method (Spencer & Weber, 1969; Lakowicz *et al.*, 1981) in the SLM 4800S spectrofluorimeter. The modulation frequencies were 6, 18 and 30 MHz. The reference compound in analyses of AEDANS lifetimes was dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] (Packard Instruments, Downers Grove, IL, U.S.A.) in ethanol (Lakowicz *et al.*, 1981). The protein concentration was 0.3–0.4 μM, i.e. the concentration of fluorophore was 1.2–1.6 μM. Excitation was at 342 nm and fluorescence was observed through a filter with 50% transmittance at 418 nm (KV418; Schott, Mainz, Germany). Tryptophan lifetimes were measured with a protein concentration of 0.4 μM, and *p*-terphenyl (Merck) in ethanol was used as reference compound (Lakowicz *et al.*, 1981). The excitation wavelength was 280 nm and fluorescence was observed through the monochromator set at 326 nm with

a bandwidth of 16 nm. The instrument was tested with quinine bisulphate in 0.05 M-H_2SO_4, which gave a lifetime within 10% of the expected value (Chen, 1974) with both phase and modulation measurements at all three modulation frequencies.

Acrylamide quenching (Eftink & Ghiron, 1976) of AEDANS and dansylcadaverine fluorescence was analysed by successive addition of small volumes (5–15 μl) of 8 M-acrylamide (Eastman–Kodak Co., Rochester, NY, U.S.A.) to 2–2.5 ml of labelled protein (0.35–0.4 μM, i.e. with a fluorophore concentration of 1.4–1.6 μM), which was continuously stirred in a 1 cm-pathlength cell. The fluorescence intensity was then measured at 25.0 ± 0.1 °C with an excitation wavelength of 340–342 nm and an emission wavelength set at the maximum of the uncorrected emission spectrum. The bandwidths in the excitation and emission channels were 4 and 8–16 nm respectively. The data were corrected for the small dilution and plotted according to the Stern–Volmer equation (Eftink & Ghiron, 1976).

Quenching of the tryptophan fluorescence of α_2M by iodide was studied essentially as described by Lehrer & Leavis (1978). Successive amounts of α_2M (0.28 μM) in 50 mM-Hepes/NaOH/0.7 M-NaI/70 μM-$Na_2S_2O_3$, pH 7.4, were added to 1.75 ml of α_2M at the same concentration in 50 mM-Hepes/NaOH/0.7 M-NaCl, pH 7.4, thus keeping the protein concentration and ionic strength constant. The analyses were done in a 1 cm-pathlength cell, the contents of which were continuously stirred during measurements. Fluorescence intensity was measured at 25.0 ± 0.1 °C with excitation and emission wavelengths of 280 and 326 nm respectively, and with corresponding bandwidths of 2 and 8 nm. The data were corrected for inner-filter effects and were plotted according to a modified Stern–Volmer equation (Lehrer, 1971).

Solvent-perturbation absorption-difference-spectroscopy studies of α_2M utilized procedures developed by Herskovitz & Laskowski (1962) and Herskovitz (1965, 1967). The final concentration of protein was 1.9–2.1 μM and that of the perturbant, ethylene glycol, was 20% (v/v). The difference spectra were measured at 25.0 ± 0.1 °C in 1 cm-pathlength double-compartment cells and were recorded with a bandwidth of 1 nm on the 0.1 absorbance scale of a Cary 219 double-beam spectrophotometer (Varian Associates, Palo Alto, CA, U.S.A.). The spectra were fitted with the model-compound data of Herskovitz & Sorensen (1968).

Protein concentrations were obtained by absorption measurements at 280 nm. The specific absorption coefficients and relative molecular masses used in the calculations have been reported previously (Curtis & Lorand, 1976; Björk & Fish, 1982; Björk *et al.*, 1985). All trypsin concentrations were active-site concentrations.

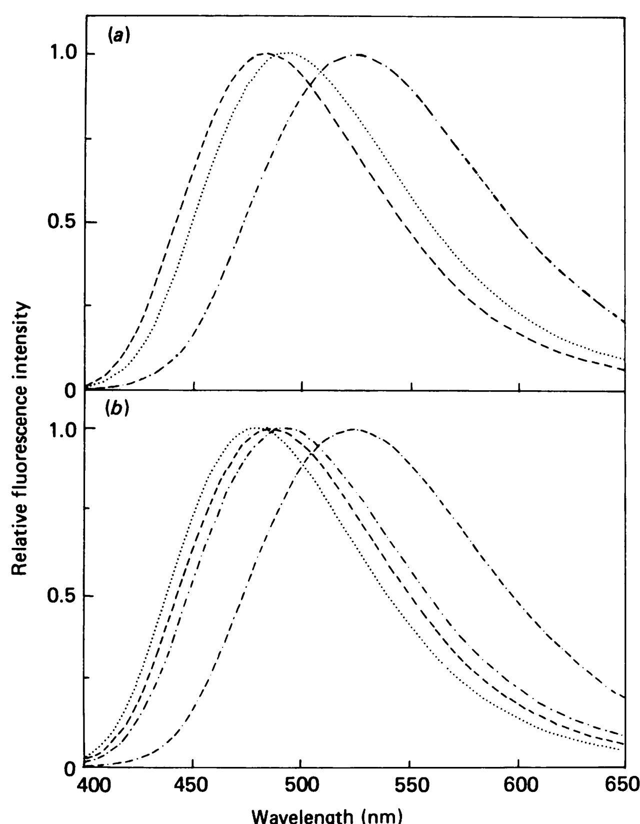

Fig. 1. Normalized corrected fluorescence emission spectra of AEDANS bound to the thiol groups of (*a*) human or (*b*) bovine α_2M liberated by prior reaction of the protein with methylamine or trypsin

Key to spectra: ······, AEDANS bound to methylamine-treated α_2M; ----, AEDANS bound to trypsin-treated α_2M; -·-·-·, AEDANS bound to methylamine-treated α_2M, followed by reaction of the labelled protein with trypsin; ·---, Ac-Cys-AEDANS. The concentrations of protein-bound AEDANS and Ac-Cys-AEDANS were 1.0–1.2 μM and 25 μM respectively, and the solvent was 50 mM-Hepes/NaOH/0.1 M-NaCl, pH 8.0. The methylamine-treated labelled bovine α_2M was allowed to react for 5 min with trypsin in a molar ratio of 1:0.7, conditions sufficient to completely convert the modified inhibitor into its fully conformationally altered form (Björk *et al.*, 1985).

RESULTS AND DISCUSSION

Changes in the thioester-bond region

The conformational changes occurring in the thioester-bond region of α_2M on reaction of the inhibitor with methylamine or trypsin were characterized by analyses of the fluorescence of a probe bound to the liberated thiol groups. Both human and bovine α_2M were studied, since different information can be obtained with the two proteins. The thiol groups were labelled with the 1,8-isomer of the fluorophore AEDANS (Hudson & Weber, 1973) by allowing the proteins to react with amine or proteinase in the presence of the labelling reagent, I-AEDANS. Human α_2M bound 4.1 and 4.2 mol of AEDANS/mol of protein when allowed to react with methylamine and trypsin respectively, whereas the corresponding values for bovine α_2M were 4.0 and 3.8 mol of AEDANS/mol of protein. Thus all four liberated thiol groups (Sottrup-Jensen *et al.*, 1980; Howard, 1981; Salvesen *et al.*, 1981) were labelled. Gradient-gel electrophoresis (Björk, 1984) showed no change of the electrophoretic mobility of bovine α_2M after labelling during the reaction with methylamine (Dangott & Cunningham, 1982), whereas all other labelled proteins migrated as the 'fast' form (Barrett *et al.*, 1979).

Binding of the fluorophore to methylamine-treated or trypsin-treated human α_2M resulted in a large blue shift of the emission and a considerably higher quantum yield,

Table 1. Emission maxima, quantum yields, fluorescence lifetimes and Stern–Volmer acrylamide quenching constants of AEDANS bound to the thiol groups of human or bovine α_2M liberated by prior reaction of the protein with methylamine or trypsin

Emission maxima and quantum yields were obtained from spectra measured as described in the legend to Fig. 1. Quenching constants were derived from the slopes of the plots in Fig. 2 (Eftink & Ghiron, 1976). The concentrations of protein-bound AEDANS and Ac-Cys-AEDANS in the lifetime analyses were 1.2–1.6 and 20 μM respectively, and the solvent was 50 mM-Hepes/NaOH/0.1 M-NaCl, pH 8.0. The reaction of the methylamine-treated labelled bovine α_2M with trypsin was done as described in the legend to Fig. 1. Abbreviations used: M-, methylamine-treated; T-, trypsin-treated; Hα_2M, human α_2M; Bα_2M, bovine α_2M; Ph., phase measurements; Mod., modulation measurements; N.D., not determined.

Protein	Emission maximum (nm)	Quantum yield	Lifetime (ns) 6 MHz Ph.	6 MHz Mod.	18 MHz Ph.	18 MHz Mod.	30 MHz Ph.	30 MHz Mod.	Quenching constant (M^{-1})
M-Hα_2M-AEDANS	493	0.17	15.5	18.0	13.1	15.9	13.2	15.3	2.8
T-Hα_2M-AEDANS	483	0.27	21.1	24.3	17.2	21.7	17.3	21.0	1.4
M-Bα_2M-AEDANS	481	0.33	21.1	25.4	16.0	21.9	14.4	20.7	1.4
T-Bα_2M-AEDANS	489	0.24	17.7	21.6	13.5	18.3	12.0	17.1	1.4
M-Bα_2M-AEDANS +trypsin	493	0.17	14.8	18.9	11.5	15.2	10.0	14.0	1.8
Ac-Cys-AEDANS	525	0.02	3.4	3.7	3.2	3.2	3.2	3.3	N.D.

compared with the data for the reference compound, Ac-Cys-AEDANS (Fig. 1*a*; Table 1). These changes were more pronounced for the trypsin-treated than for the methylamine-treated inhibitor. Similarly, the fluorescence lifetime was considerably longer for the protein-bound AEDANS than for Ac-Cys-AEDANS and also longer for the fluorophore bound to trypsin-treated α_2M than for that bound to the methylamine-treated inhibitor (Table 1). However, for both proteins the lifetimes obtained from the phase shift and demodulation at a single modulation frequency differed from each other and also from the values obtained at the other frequencies, indicative of multiexponential decay. The origin of this heterogeneity was not investigated further.

AEDANS bound to methylamine- or trypsin-treated bovine α_2M also had an emission maximum at a shorter wavelength and a markedly higher quantum yield than Ac-Cys-AEDANS (Fig. 1*b*; Table 1). However, in contrast with the human protein, these effects were smaller for the trypsin-treated than for the methylamine-treated inhibitor. Reaction of the labelled methylamine-treated bovine α_2M with trypsin increased the wavelength of the emission maximum and decreased the quantum yield to values that were only slightly different from those of AEDANS bound to α_2M that had been allowed to react with trypsin directly (Fig. 1*b*; Table 1). Again, lifetime measurements supported these data (Table 1). The lifetime of the protein-bound AEDANS thus was markedly longer than that of Ac-Cys-AEDANS. Moreover, AEDANS bound to trypsin-treated bovine α_2M had a shorter lifetime than that bound to the methylamine-treated inhibitor, and reaction of the latter with trypsin caused an appreciable decrease of the lifetime. A lifetime heterogeneity similar to that observed for AEDANS bound to human α_2M was found also for the fluorophore bound to the bovine inhibitor.

The acrylamide quenching of the fluorescence of AEDANS bound to human α_2M was larger for the methylamine-treated than for the trypsin-treated inhibitor (Fig. 2*a*; Table 1). The linear Stern–Volmer plots obtained for both proteins indicate that the fluorophores are approximately equally accessible to the quencher (Eftink & Ghiron, 1976). In contrast with human α_2M, the fluorescence of AEDANS bound to methylamine-treated and trypsin-treated bovine α_2M was quenched to approximately the same extent (Fig. 2*b*; Table 1). Moreover, the fluorophore bound to the methylamine-

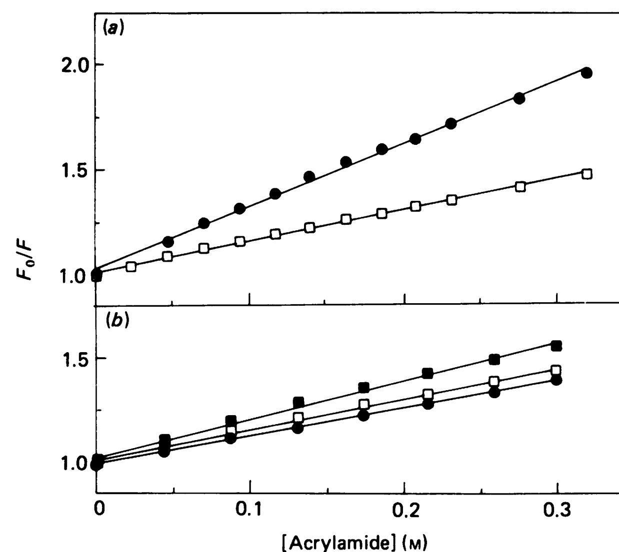

Fig. 2. Acrylamide quenching of the fluorescence of AEDANS bound to the thiol groups of (*a*) human or (*b*) bovine α_2M liberated by prior reaction of the protein with methylamine or trypsin

●, AEDANS bound to methylamine-treated α_2M; □, AEDANS bound to trypsin-treated α_2M; ■, AEDANS bound to methylamine-treated α_2M, followed by reaction of the labelled protein with trypsin. The concentration of protein-bound AEDANS was 1.4–1.6 μM, and the solvent was 50 mM-Hepes/NaOH/0.1 M-NaCl, pH 8.0. Methylamine-treated labelled bovine α_2M was allowed to react with trypsin as described in the legend to Fig. 1. F_0 and F, fluorescence intensity in the absence or presence of quencher respectively.

treated α_2M was rendered only slightly more accessible to the quencher by reaction of the labelled inhibitor with trypsin (Fig. 2*b*; Table 1). Lifetime measurements in 0.3 M-acrylamide showed that the quenching of the protein-bound AEDANS was predominantly collisional in nature, but with a contribution from static quenching (Lakowicz, 1983).

Taken together the data indicate that AEDANS bound to either methylamine-treated or trypsin-treated human or bovine α_2M is in a hydrophobic environment. The protein-bound fluorophore thus has a blue-shifted emission spectrum, a higher quantum yield and a longer lifetime than the reference compound Ac-Cys-AEDANS. The thiol group liberated by thioester-bond cleavage therefore is most likely partially buried in all forms of the inhibitor. A partially shielded localization of the thioester-bond region is also indicated by the limited reactivity of this bond with large primary amines (Larsson & Björk, 1984).

The analyses of the labelled human α_2M allow only a comparison of the local environments of the thiol groups in the methylamine- and trypsin-treated forms of the inhibitor, both of which have undergone similar, major, conformational changes. The differences in fluorescence emission and lifetime of AEDANS bound to the two forms suggest that the liberated thiol group is in more hydrophobic surroundings in human α_2M that has bound trypsin than in the methylamine-treated inhibitor. The fluorophore bound to trypsin-treated α_2M is also less accessible to quenching. Since the conformational changes that have occurred in the two forms of α_2M are highly similar (Björk & Fish, 1982; Gonias *et al.*, 1982; Dangott *et al.*, 1983), the different environments may be due to a close proximity of the liberated thiol group to the bound proteinase, in agreement with a previous similar proposal (Pochon *et al.*, 1983).

In contrast with human α_2M, cleavage of the thioester bonds of bovine α_2M by amines only minimally perturbs the conformation of this protein. The methylamine-treated bovine inhibitor also retains the ability to bind proteinases and undergoes a similar conformational change as intact α_2M in this reaction (Dangott & Cunningham, 1982; Björk *et al.*, 1985). The studies of the labelled bovine inhibitor thus allow conclusions on the nature of the local changes in the thioester-bond region that occur during the major conformational change involved in proteinase binding. The differences in fluorescence emission and lifetime between AEDANS bound to methylamine- and trypsin-treated bovine α_2M suggest that the fluorophore is in a less hydrophobic environment when bound to the trypsin-treated form. This conclusion is supported by the analyses of the reaction of the methylamine-treated inhibitor with trypsin, which produced similar effects. The major conformational change of bovine α_2M accompanying the binding of proteinases thus involves transfer of the thioester-bond region of the protein to more polar surroundings. However, this change apparently does not lead to an increased exposure of the thioester region at the surface of the protein, since the fluorescence of all three forms of the labelled bovine α_2M was quenched to approximately the same extent by acrylamide. The similarity of the conformational changes induced by proteinases in human and bovine α_2M (Björk *et al.*, 1985) suggests that a similar change of the environment of the thioester-bond region also occurs in the human inhibitor.

Changes around the transglutaminase cross-linking site

Plasma transglutaminase (Factor XIIIa) has been shown to catalyse the incorporation of dansylcadaverine into human α_2M at predominantly one specific glutamine residue, located about ten residues *N*-terminal to the primary sites of cleavage in the 'bait' region (Mortensen *et al.*, 1981; Sottrup-Jensen *et al.*, 1984). A small amount ($\sim 5\%$) of the bound fluorophore is incorporated at the adjacent glutamine residue. In our work, human α_2M bound 4.5 mol of dansylcadaverine/mol of protein, indicating that approximately one molecule of the fluorophore was linked to each subunit. No change of the electrophoretic mobility of the protein was observed after labelling.

Binding of dansylcadaverine to human α_2M shifted the fluorescence emission maximum to a shorter wavelength and increased the quantum yield (Fig. 3; Table 2). Reaction of the labelled inhibitor with methylamine or trypsin further decreased the wavelength of maximum emission and increased the quantum yield (Fig. 3; Table 2). These changes were more pronounced for trypsin-treated α_2M. Only minimal quenching of the dansylcadaverine fluorescence was observed for all forms of the inhibitor up to an acrylamide concentration of 0.5 M, whereas the fluorescence of free dansylcadaverine was appreciably quenched (result not shown).

These results show that binding of dansylcadaverine to the transglutaminase cross-linking site of α_2M shields the fluorophore from solvent. The binding region thus presumably is located some distance from the surface of the protein. However, the comparatively low increase in quantum yield of the bound inhibitor suggests that the surroundings of the site are only moderately hydrophobic. Reaction of the labelled inhibitor with methylamine or trypsin induces conformational changes that

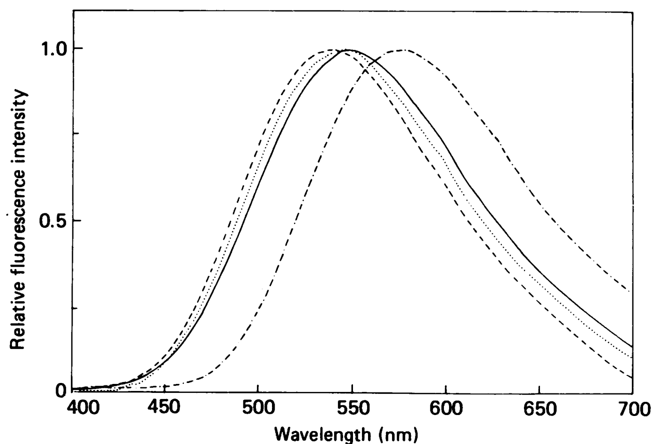

Fig. 3. Normalized corrected fluorescence emission spectra of dansylcadaverine bound to the transglutaminase cross-linking site in human α_2M, before and after reaction of the labelled protein with methylamine or trypsin

——, Dansylcadaverine–α_2M; ······, dansylcadaverine–α_2M treated with methylamine; ----, dansylcadaverine–α_2M treated with trypsin; ·–·–·, free dansylcadaverine. The concentrations of protein-bound and free dansylcadaverine were 1.5–1.6 and 20 μM respectively, and the solvent was 50 mM-Hepes/NaOH/0.1 M-NaCl, pH 8.0. Dansylcadaverine–α_2M was allowed to react for 2 h with 100 mM-methylamine or for 5 min with trypsin in a molar ratio of inhibitor to enzyme of 1:2.2.

Table 2. Emission maxima and quantum yields of dansylcadaverine bound to the transglutaminase cross-linking site in human α_2M, before and after reaction of the labelled protein with methylamine or trypsin

The values were obtained from spectra measured as described in the legend to Fig. 3.

Protein	Emission maximum (nm)	Quantum yield
Dansylcadaverine–α_2M	548	0.05
Dansylcadaverine–α_2M + methylamine	542	0.07
Dansylcadaverine–α_2M + trypsin	537	0.08
Free dansylcadaverine	575	0.03

render the cross-linking region more hydrophobic, thus possibly further burying the fluorophore. In contrast with the gross conformational change in the inhibitor (Gonias *et al.*, 1982; Björk & Fish, 1982; Dangott *et al.*, 1983), these local changes are somewhat different for the methylamine- and trypsin-treated inhibitors. The environment of the cross-linking site thus is more apolar after the reaction with trypsin. This difference may be an effect of the cleavage of the adjacent 'bait' region that occurs only on reaction of α_2M with proteinases. Conversely, the conformational change around the transglutaminase cross-linking site that is induced when α_2M reacts with amines without proteolytic cleavage may extend also to the 'bait' region.

Changes around aromatic amino acids

The observed fluorescence lifetime of tryptophan in human α_2M increased on reaction of the inhibitor with methylamine or trypsin (Table 3), indicating transfer of tryptophan residues to a more hydrophobic environment. No difference between methylamine- and trypsin-treated α_2M was apparent.

Perturbation of the u.v. absorption of human α_2M in

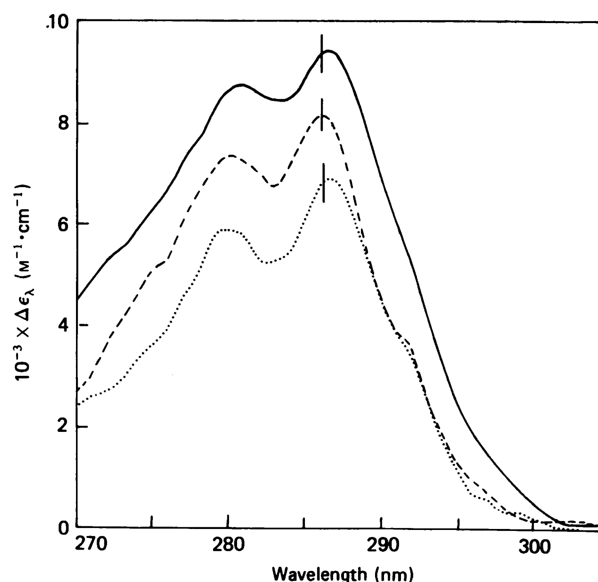

Fig. 4. Solvent-perturbation absorption-difference spectra, due to 20% ethylene glycol, of human α_2M before and after reaction of the protein with methylamine or trypsin

——, α_2M; · · · · · ·, α_2M treated with methylamine; – – – –, α_2M treated with trypsin. The buffer was 50 mM-Hepes/NaOH/0.1 M-NaCl, pH 7.4. α_2M was allowed to react for 7 h with 200 mM-methylamine or for 5 min with trypsin in a molar ratio of inhibitor to enzyme of 1:2.2. The vertical bars are the S.D. values (for intact and methylamine-treated α_2M; $n = 3$) or the range (for trypsin-treated α_2M; $n = 2$).

the aromatic wavelength region by 20% ethylene glycol indicated that about 30% of both the tyrosine and tryptophan residues of the protein are accessible to solvent (Fig. 4; Table 3). No change in the exposure of tyrosine residues on reaction of the inhibitor with methylamine or trypsin could be ascertained. However, five or six fewer tryptophan residues per α_2M tetramer were accessible to solvent after either of these reactions, i.e. the fraction of exposed tryptophan residues decreased

Table 3. Fluorescence lifetimes and results of analyses by solvent-perturbation absorption-difference spectroscopy and by iodide quenching of the tryptophan fluorescence of human α_2M before and after reaction of the protein with methylamine or trypsin

Lifetimes were obtained by phase measurements at high modulation frequencies, since both phase measurements at lower frequencies and modulation measurements of short lifetimes are subject to large errors (Lakowicz, 1983). The solvent in these analyses was 50 mM-Hepes/NaOH/0.1 M-NaCl, pH 7.4. The reactions with methylamine or trypsin were done as described in the legend to Fig. 4. The solvent-perturbation and iodide-quenching data were calculated from Figs. 4 and 5 respectively (Herskovitz & Sorensen, 1968; Lehrer, 1971).

	Lifetime (ns)		Solvent perturbation				Iodide quenching	
			Tyr exposed		Trp exposed			
Protein	18 MHz	30 MHz	No.	%*	No.	%*	Quenching constant (M^{-1})	Accessible tryptophan fluorescence (%)
α_2M	1.35	1.19	77	34	13	29	23	25
α_2M + methylamine	1.78	1.58	69	31	7	16	34	18
α_2M + trypsin	1.88	1.70	73	33	8	18	34	17

* Calculated from a total of 224 tyrosine and 44 tryptophan residues in α_2M tetramer (Sottrup-Jensen *et al.*, 1984).

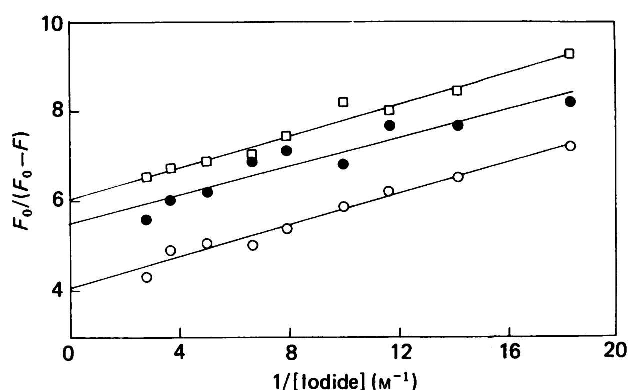

Fig. 5. Iodide quenching of the tryptophan fluorescence of human α_2M before and after reaction of the protein with methylamine or trypsin

○, α_2M; ●, α_2M treated with methylamine; □, α_2M treated with trypsin. α_2M was allowed to react with methylamine or trypsin as described in the legend to Fig. 4. F_0 and F, fluorescence intensity in the absence or presence of quencher respectively.

to 16–18%. Iodide quenching studies showed that about 25% of the tryptophan fluorescence of human α_2M was accessible to the quencher (Fig. 5; Table 3), in good agreement with the results obtained by solvent-perturbation difference spectroscopy. Moreover, a similar decrease of the accessible tryptophan fluorescence, to 17–18%, was observed on reaction of the inhibitor with either methylamine or trypsin.

Taken together these data indicate that one or two tryptophan residues in each α_2M monomer are buried on reaction of the inhibitor with either amines or proteinases, with no discernible change in the exposure of tyrosine residues. This conclusion thus strongly reinforces a previous tentative interpretation of the absorption and fluorescence changes accompanying these reactions (Björk & Fish, 1982).

General conclusions

The spectroscopic analyses in the present work are consistent with an extensive conformational change of α_2M on reaction with amines or proteinases, affecting both the thioester-bond region, the transglutaminase cross-linking site, and thus possibly also the adjacent 'bait' region, and the environment around one or two tryptophan residues. Such a pronounced change of conformation is also indicated by the hydrodynamic and c.d. changes that accompany these reactions (Gonias *et al.*, 1982; Björk & Fish, 1982; Dangott *et al.*, 1983). Although the gross conformational changes induced in α_2M by amines and proteinases are very similar (Gonias *et al.*, 1982; Björk & Fish, 1982; Dangott *et al.*, 1983), the present studies furthermore show that certain local perturbations of the structure of the protein are different in the two reactions. These local differences may partly be due to the presence of the bound proteinase or cleavage of the 'bait' region.

Recently, a model for α_2M structure and function has been proposed (Feldman *et al.*, 1985). Although largely speculative, it is consistent with most available data and provides an appropriate basis for further studies of the inhibitor. Essentially, an α_2M half-molecule is pictured as consisting of a ring with four arms projecting from one side, thus resembling a royal crown. Each monomer in the half-molecule has two such arms, one long and one short. The ring portions of the two half-molecules interact to form the whole tetrameric molecule. An essential feature of the model is that trapping of the proteinase may occur by slight inward movement of the two long arms in the half-molecule.

Our data are in agreement with several aspects of this model. Movement of the 'trapping' arm, which presumably also occurs when α_2M reacts with amines, may thus transfer the thioester-bond region, suggested to be located at the base of this arm (Feldman *et al.*, 1985), to a more hydrophilic environment, while preserving the limited access to this region. The proposed location of the thioester bond is also consistent with the liberated thiol group being close to the bound proteinase. Moreover, a reorientation of the polypeptide chains in the contact region between the two half-molecules concomitant with trap closure, a structural change proposed to explain the extensive change of conformation (Feldman *et al.*, 1985), may perturb the environment of both the transglutaminase cross-linking site and certain tryptophan residues. A further inference from our data is that the site modified by plasma transglutaminase is unlikely to be involved in binding of the α_2M–proteinase complex to its cellular receptor, as indicated by previous work (Van Leuven *et al.*, 1981), since this site is buried rather than exposed on reaction with proteinases. The apparent role of transglutaminase activity in receptor-mediated endocytosis of α_2M–proteinase complexes into cells (Davies *et al.*, 1980) most likely is exerted through other mechanisms (Davies & Murtaugh, 1984; Van Leuven, 1984).

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REFERENCES

Barrett, A. J. & Starkey, P. M. (1973) Biochem. J. **133**, 709–724
Barrett, A. J., Brown, M. A. & Sayers, C. A. (1979) Biochem. J. **181**, 401–418
Björk, I. (1984) Biochem. Biophys. Res. Commun. **118**, 691–695
Björk, I. (1985) Biochem. J. **231**, 451–457
Björk, I. & Fish, W. W. (1982) Biochem. J. **207**, 347–356
Björk, I., Larsson, L.-J., Lindblom, T. & Raub, E. (1984) Biochem. J. **217**, 303–308
Björk, I., Lindblom, T. & Lindahl, P. (1985) Biochemistry **24**, 2653–2660
Bridges, J. W. (1981) in Standards in Fluorescence Spectroscopy (Miller, J. N., ed.), pp. 75–76, Chapman and Hall, London and New York
Chase, T. & Shaw, E. (1970) Methods Enzymol. **19**, 20–27
Chen, R. F. (1968) Anal. Biochem. **25**, 412–416
Chen, R. F. (1974) Anal. Biochem. **57**, 593–604
Curtis, C. G. & Lorand, L. (1976) Methods Enzymol. **45**, 177–191
Dangott, L. J. & Cunningham, L. W. (1982) Biochem. Biophys. Res. Commun. **107**, 1243–1251
Dangott, L. J., Puett, D. & Cunningham, L. W. (1983) Biochemistry **22**, 3647–3653
Davies, P. J. A. & Murtaugh, M. P. (1984) Mol. Cell. Biochem. **58**, 69–77

Davies, P. J. A., Davies, D. R., Levitzki, A., Maxfield, F. R., Milhand, P., Willingham, M. C. & Pastan, I. H. (1980) Nature (London) **283**, 162–167
Eccleston, E. D. & Howard, J. B. (1985) J. Biol. Chem. **260**, 10169–10176
Eftink, M. R. & Ghiron, C. A. (1976) J. Phys. Chem. **80**, 486–493
Feldman, S. R., Gonias, S. L. & Pizzo, S. V. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 5700–5704
Ganrot, P. O. (1966) Acta Chem. Scand. **20**, 2299–2300
Gonias, S. L. & Pizzo, S. V. (1983) Biochemistry **22**, 536–546
Gonias, S. L., Reynolds, J. A. & Pizzo, S. V. (1982) Biochim. Biophys. Acta **705**, 306–314
Hall, P. K. & Roberts, R. C. (1978) Biochem. J. **173**, 27–38
Harpel, P. (1973) J. Exp. Med. **138**, 508–521
Harpel, P. (1976) Methods Enzymol. **45**, 639–652
Herskovitz, T. T. (1965) J. Biol. Chem. **240**, 628–638
Herskovitz, T. T. (1967) Methods Enzymol. **11**, 748–775
Herskovitz, T. T. & Laskowski, M. Jr. (1962) J. Biol. Chem. **237**, 2481–2492
Herskovitz, T. T. & Sorensen, M. (1968) Biochemistry **7**, 2523–2532
Howard, J. B. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 2235–2239
Hudson, E. N. & Weber, G. (1973) Biochemistry **12**, 4154–4161
Jones, J. M., Creeth, J. M. & Kekwick, R. A. (1972) Biochem. J. **127**, 187–197
Lakowicz, J. R. (1983) Principles of Fluorescence Spectroscopy, pp. 79–86, Plenum Press, New York
Lakowicz, J. R., Cherek, H. & Balter, A. (1981) J. Biochem. Biophys. Methods **5**, 131–146
Larsson, L.-J. & Björk, I. (1984) Biochemistry **23**, 2802–2807
Larsson, L.-J., Olson, S. T. & Björk, I. (1985) Biochemistry **24**, 1585–1593
Lehrer, S. S. (1971) Biochemistry **10**, 3254–3263
Lehrer, S. S. & Leavis, P. C. (1978) Methods Enzymol. **49**, 222–236
Lorand, L. & Gotoh, T. (1970) Methods Enzymol. **19**, 770–782
Mortensen, S. B., Sottrup-Jensen, L., Hansen, H. F., Rider, D., Petersen, T. E. & Magnusson, S. (1981) FEBS Lett. **129**, 314–317
Pochon, F., Favaudon, V., Tourbez-Perrin, M. & Bieth, J. (1981) J. Biol. Chem. **256**, 547–550
Pochon, F., Favaudon, V. & Bieth, J. (1983) Biochem. Biophys. Res. Commun. **111**, 964–969
Salvesen, G. S. & Barrett, A. J. (1980) Biochem. J. **187**, 695–701
Salvesen, G. S., Sayers, C. A. & Barrett, A. J. (1981) Biochem. J. **195**, 453–461
Schramm, H. J. & Schramm, W. (1982) Hoppe-Seyler's Z. Physiol. Chem. **363**, 803–812
Sottrup-Jensen, L., Petersen, T. E. & Magnusson, S. (1980) FEBS Lett. **121**, 275–279
Sottrup-Jensen, L., Lønblad, P. B., Stepanik, T. M., Petersen, T. E., Magnusson, S. & Jörnvall, H. (1981*a*) FEBS Lett. **127**, 167–173
Sottrup-Jensen, L., Petersen, T. E. & Magnusson, S. (1981*b*) FEBS Lett. **128**, 123–126
Sottrup-Jensen, L., Stepanik, T. M., Kristensen, T., Wierzbicki, D. M., Jones, C. M., Lønblad, P. B., Magnusson, S. & Petersen, T. E. (1984) J. Biol. Chem. **259**, 8318–8327
Spencer, R. D. & Weber, G. (1969) Ann. N.Y. Acad. Sci. **158**, 361–376
Strickland, D. K. & Bhattacharya, P. (1984) Biochemistry **23**, 3115–3124
Swenson, R. P. & Howard, J. B. (1979) J. Biol. Chem. **254**, 4452–4456
Van Leuven, F. (1984) Mol. Cell. Biochem. **58**, 121–128
Van Leuven, F., Cassiman, J.-J. & Van den Berghe, H. (1979) J. Biol. Chem. **254**, 5155–5160
Van Leuven, F., Cassiman, J.-J. & Van den Berghe, H. (1981) J. Biol. Chem. **256**, 9016–9022
Wu, K., Wang, D. & Feinman, R. D. (1981) J. Biol. Chem. **256**, 10409–10414

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