Separation and Localization of the Four Cysteine-949 Residues in Human α_2-Macroglobulin Using Fluorescence Energy Transfer†

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Received February 26, 1990; Revised Manuscript Received May 10, 1990

ABSTRACT: By use of the intermediate form (I-form) [Gettins, Crews, & Cunningham (1989) *Biochemistry 28*, 5613–5618], α_2-macroglobulin can be specifically labeled with fluorescent probes in a manner that allows the determination of the topology of the four thiol ester derived Cys949 residues within this large tetrameric protease inhibitor. Freshly prepared I-form α_2-macroglobulin was reacted with 5-[[2-[(iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonate (1,5-I-AEDANS) to produce α_2-macroglobulin specifically and stoichiometrically labeled with 1,5-AEDANS (donor) at the two Cys949 SH groups in the first protease interaction site. Upon subsequent reaction of this labeled species with chymotrypsin, the remaining two bait regions and thiol ester linkages were opened, generating two free SH groups on the two Cys949 residues in the second protease interaction site. These SH groups were specifically and stiochiometrically labeled with 5-(iodoacetamido)fluorescein (acceptor). Fluorescence energy transfer from donor to acceptor results in 82% loss of AEDANS fluorescence intensity. By use of an $R_0(2/3)$ value of 43.5 Å, calculated from the spectral parameters of this system, an $R(2/3)$ separation between donor and acceptor of 33.9 Å was calculated. From fluorescence anisotropy measurements of both donor and acceptor attached to α_2-macroglobulin, upper and lower limits on the separation of 43.4 and 26.1 Å, respectively, were calculated. These separations, small in the context of the α_2-macroglobulin tetramer, which has approximate dimensions of 190 × 90 × 90 Å, severely restrict the possible locations of the four Cys949 residues. These residues, and the bait regions which are close to them, must be centrally located at the bottom of the deep cavities that constitute the two protease traps.

Human α_2-macroglobulin (α_2M)[1] is a very broad specificity plasma protease inhibitor (Barret & Starkey, 1973) that is also capable of interacting with certain growth factors such as PDGF, basic FGF, and β-TGF (Huang et al., 1984, 1988; O'Connor-McCourt & Wakefield, 1987; Dennis et al., 1989). It is a tetramer of identical 180-kDa subunits, which are organized as noncovalently associated disulfide-linked dimers (Hyldgaard Jensen & Sottrup-Jensen, 1986). Although many chemical and physical methods have been used to examine human α_2-macroglobulin and related proteins from other species [see Sottrup-Jensen (1989) for a recent review], most of the details of the overall three-dimensional structure of human α_2-macroglobulin have been obtained from electron microscopy studies (Schramm & Schramm, 1982; Tapon-Bretaudière et al., 1985; Delain et al., 1988; Arakawa et al., 1989). While the conclusions from these electron microscopy studies differ in regard to the structure of the native protein, perhaps reflecting the protein's conformational flexibility and consequent deformability upon sample preparation, there is general agreement as to the overall shape and protein density distribution of the form that has reacted either with protease or with methylamine. Reaction of α_2-macroglobulin with either results in scission of an internal thiol ester present in each subunit and a major conformational rearrangement that exposes a receptor recognition site. The reaction with protease also results in the protease's sequestration. In gross outline, the reacted form of α_2-macroglobulin appears to have the shape of the cyrillic letter H, with a length of 190 Å and a diameter of 90 Å (Schramm & Schramm, 1982; Feldman et al., 1985).

The two structural elements of α_2-macroglobulin critical to the efficient sequestration of proteases are the thiol ester and the bait region. Cleavage of the bait region by protease results in thiol ester opening, and these two alterations in the primary structure bring about the conformational rearrangements necessary for both receptor recognition site exposure and trapping of the protease. It has recently been demonstrated using NMR spectroscopy that the thiol ester and the bait region are located close to one another (Gettins et al., 1988). However, information on the location of the four thiol esters within the tetramer is less definitive, though we have demonstrated by EPR spectroscopy of uniformly spin-labeled α_2M that no pair of SH-linked nitroxides is closer together than 20 Å (Gettins et al., 1988). The finding that the carboxyl moiety generated upon opening of the thiol ester, subsequent to reaction of α_2-macroglobulin with protease, is frequently involved in a covalent cross-link to the protease through the ϵ-amino group of lysine strongly suggests a location on the internal face of the inhibitor for the residues that form the thiol

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‖ Lucille P. Markey Scholar in Biomedical Science and supported by the Lucille P. Markey Foundation.

[1] Abbreviations: α_2M, α_2-macroglobulin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; PROXYL, 2,2,5,5-tetramethylpyrrolidine-1-oxyl; I-form, intermediate migratory form; 5-IAF, 5-(iodoacetamido)fluorescein; PDGF, platelet-derived growth factor; β-TGF, β-transforming growth factor; FGF, fibroblast growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 1,5-I-AEDANS, 5-[[2-[(iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonate; PMSF phenylmethanesulfonyl fluoride.

0006-2960/90/0429-7747$02.50/0

ester. An electron microscopy study in which ferritin was coupled via avidin and biotin to the liberated SH groups concluded that the SH groups are distally located at the ends of the four arms of the molecule (Schramm & Witke, 1988). This is the region of α_2M that has been identified with the receptor recognition site and has been shown to be a discrete domain that does not include either the thiol ester or the bait region (Van Leuven et al., 1986; Enghild et al., 1989). Also such a peripheral location for the thiol esters and bait regions is less easy to reconcile with the high efficiency of protease trapping than a more central location. In an attempt to further localize the thiol ester residues and in particular to distinguish between a location close to the central bar of protein density and a much more peripheral location, we have employed fluorescence energy transfer to measure the separation between Cys949 residues in the first protease binding site and those located in the second protease binding site. For these measurements, a dansyl chromophore was covalently attached to each of the two free SH groups in the first protease binding site, and a fluorescein chromophore was attached to each of the two SH groups in the second protease binding site. This specific labeling used the intermediate form of α_2-macroglobulin that has been described previously (Gettins et al., 1989), which permits sequential labeling of the first and second pair of thiol ester derived free SH groups. A large amount of nonradiative energy transfer is found in this system, which is consistent only with a central location for the thiol esters, and in turn for the four bait regions.

Materials and Methods

Purification of α_2-Macroglobulin. Human α_2M was purified from recently expired plasma (American Red Cross, Nashville Region) by zinc–chelate chromatography, as described previously (Roche et al., 1989). The purity of each preparation was checked by electrophoresis under nondenaturing conditions and also in the presence of SDS. Nondenaturing PAGE was performed in 5% acrylamide slabs (Davis, 1964). SDS-PAGE was carried out in 7.5% slab gels according to the procedure of Laemmli (1970). α_2M concentrations were determined spectrophotometrically using $E^{1\%} = 8.9$ (Hall & Roberts, 1978; Barrett et al., 1979) and a molecular weight of 716 000 based upon the primary structure (Sottrup-Jensen et al., 1984).

All samples for SDS–PAGE were denatured and reduced for 45 min at 37 °C in buffer containing 2% SDS and 0.8% dithiothreitol.

Preparation of Intermediate-Form α_2-Macroglobulin. I-Form α_2M was prepared as previously described (Gettins et al., 1989). Sixty milligrams of α_2M was reacted with 12 mL of chymotrypsin–Sepharose in 50 mM HEPES, 0.1 M NaCl, and 2 mM EDTA, pH 7.5. The course of the reaction was followed by assay of free SH groups using DTNB. After 3 h of reaction, 1.91 mol of SH was present per mole of α_2M tetramer, and an additional 1.85 mol of SH could be released by incubation with 0.1 M CH_3NH_2 for 30 min. The reaction was halted by centrifugation and filtration through a 0.2-μm filter. A yield of 68% of I-form α_2M was obtained.

Preparation and Characterization of Fluorescently Labeled α_2-Macroglobulin Species. The dansyl chromophore was covalently attached to the two free SH groups (Cys949) of I-form α_2M by reaction with 1,5-I-AEDANS. I-form α_2M (4.6 μM) was reacted with 122 μM 1,5-I-AEDANS in 0.1 M sodium phosphate buffer at pH 7.0 for 25 min. The sample was then diluted 4-fold and concentrated to one-tenth volume prior to dialysis against 1000 volumes of 0.1 M phosphate buffer. The sample was spun at 87000*g* for 30 min in a Beckman TL100 ultracentrifuge prior to recording the UV/visible spectrum on a Cary 219 spectrophotometer for the purposes of quantitation of incorporated label. The number of dansyl groups per α_2M tetramer was calculated from the relative absorbances at 342 and 280 nm. By use of an extinction coefficient of 6.3×10^3 M^{-1} cm^{-1} at 342 nm for the dansyl chromophore (Hudson & Weber, 1973) and the extinction coefficient given above for α_2M at 280 nm, together with a correction for absorption at 280 nm by AEDANS, a stoichiometry of 2.1 AEDANS groups per tetramer was calculated.

The fluorescein group was introduced by reaction of α_2M with 5-(iodoacetamido)fluorescein (Molecular Probes, Eugene, OR). Three separate fluorescein-labeled species were prepared. Dansylated I-form α_2M, prepared as above, was reacted with 1.2 equiv of α-chymotrypsin for 10 min at room temperature in the presence of 1 mM 5-IAF. The chymotrypsin was then inhibited with 0.5 mM PMSF and the labeling of the liberated SH groups allowed to proceed at 4 °C in the dark for 18 h (Trayer & Trayer, 1988). The reaction mixture was then diluted and reconcentrated 3 times in an ultrafiltration cell to remove most of the unreacted 5-IAF prior to dialysis. A second form of α_2M was produced by using methylamine to liberate SH groups from the remaining two thiol esters of dansylated I-form α_2M rather than through the use of protease. For this sample, dansylated I-form α_2M was reacted with 0.2 M methylamine at pH 8.0 for 60 min at room temperature. These conditions are sufficient to react completely with the thiol esters of α_2M (Strickland & Bhattacharya, 1984). The pH was readjusted to pH 7.0 and unreacted methylamine removed by three cycles of dilution and reconcentration in an Amicon ultrafiltration cell. The liberated SH groups were then labeled with 1 mM 5-IAF at 4 °C for 18 h. Unreacted reagent was removed as for the first sample. The third fluorescein-labeled α_2M species was a control sample in which the two SH groups liberated in I-form α_2M were labeled with iodoacetamide followed by 5-IAF labeling at the second pair of SH groups in an identical manner with the first sample. Prior to spectrophotometric quantitation of the stoichiometry of labeling, all three samples were spun in a Beckman TL100 ultracentrifuge at 87000*g* for 30 min. The concentration of fluorescein label was determined from the absorbance at 494 nm using an extinction coefficient of 7.13×10^4 M^{-1} cm^{-1} (Eshaghpour et al., 1980). The protein concentration was determined from the absorbance at 280 nm with corrections for the contributions at this wavelength from both the dansyl groups (where appropriate) and the fluorescein groups. Stoichiometries of 2.16:1, 2.27:1, 2.21:1 were obtained for dansylfluorescein (protease-treated) α_2M, dansylfluorescein (methylamine-treated) α_2M, and acetamide–fluorescein (protease-treated) α_2M respectively. (See Results for a discussion of the specificity of labeling.)

Fluorescence Measurements. Steady-state fluorescence emission and excitation spectra were recorded on an ISS Greg 200 fluorometer operated in analog mode. An excitation/emission bandwidth of 6 nm was typically employed. A plot of correction factor against wavelength for this system gave a nearly constant value of 1.0–1.1 over the wavelength region from 400 to 500 nm. Spectra are therefore reported uncorrected.

DTNB Assay. The appearance of sulfhydryl groups was measured by reaction with DTNB at 410 nm (Larsson & Björk, 1984).

Materials. Chymotrypsin–Sepharose was prepared according to the method of Gettins et al. (1989). 1,5-I-AE-

DANS was purchased from Aldrich Chemical Co., 5-IAF was from Molecular Probes, Eugene, OR, and DTNB was from Sigma Chemical Co.

Calculation of R_0 and Separation between the Donor/Acceptor Pair. The efficiency of nonradiative energy transfer from donor to acceptor (E) is related to the separation between their transition dipoles according to the relationship:

$$R = R_0(1/E - 1)^{1/6} \quad (1)$$

R_0 is the separation for 50% efficiency of transfer (Förster, 1965) and is related to the properties of the two chromophores by eq 2, where J is the spectral overlap integral, n is the

$$R_0 = (9.79 \times 10^3)(J\kappa^2 n^{-4}\Phi_D)^{1/6}\ \text{Å} \quad (2)$$

refractive index of the medium, κ^2 is an orientation factor that is determined by the relative orientation of the two chromophores and their degrees of motional freedom, and Φ_D is the quantum yield of the donor. J is calculated from the fluorescence emission spectrum of dansylated I-form α_2M and the absorption spectrum of fluorescein-labeled α_2M and has units of M^{-1} cm^3. The quantum yield of the donor was calculated by reference to a solution of quinine sulfate, using a quantum yield for the latter of 0.55 (Melhuish, 1964).

Calculation of $\kappa^2_{\text{max and min}}$ from Fluorescence Anisotropy Measurements. For a donor/acceptor pair that have isotropic rotation on the time scale of the fluorescent lifetimes, a value of $^2/_3$ can be used for κ^2. However, the range of possible values for κ^2 is 0–4, and, therefore, it is important to be able to put upper and lower limits on the orientation factor and consequently on R_0. Dale et al. (1979) have shown that fluorescence anisotropy measurements of both donor and acceptor fluorophores can be used to limit the possible upper and lower values of the orientation factor. Anisotropies were determined by recording emission spectra over a narrow wavelength range for the two control samples containing either dansyl alone or fluorescein alone, using polarizing filters and recording four sets of spectra for each, using horizontally polarized excitation and recording emission, and using either horizontal or vertical polarizers (I_{HH} and I_{HV}, respectively) and vertically polarized excitation with either horizontal or vertical polarizers for detection (I_{VH} or I_{VV}, respectively). The ratio of I_{HH}/I_{HV} corrects for instrument bias (Kirby, 1971). The anisotropies are then given by eq 3, where $I_{VV}^* = I_{VV}I_{HH}/I_{HV}$. Determination of the anisotropies was over the wavelength region 470–510 nm for the donor, with excitation at 365 nm, and from 510 to 530 nm for the acceptor, with excitation at 485 nm, and gave constant values over these regions. The average depolarization factor, $\langle d \rangle$, is related to the anisotropy by

$$A = (I_{VV}^* - I_{VH})/(I_{VV}^* + 2I_{VH}) \quad (3)$$

$$\langle d \rangle = A/0.4 = \langle d^x \rangle^2 \quad (4)$$

Maximum and minimum values of $\langle \kappa^2 \rangle$ are then given (Dale et al., 1979) by

$$\langle \kappa^2 \rangle_{max} = {}^2/_3(1 + \langle d_{D^x} \rangle + \langle d_{A^x} \rangle + 3\langle d_{D^x} \rangle \langle d_{A^x} \rangle) \quad (5)$$

$$\langle \kappa^2 \rangle_{min} = {}^2/_3[1 - (\langle d_{D^x} \rangle + \langle d_{A^x} \rangle)/2] \quad (6)$$

From these values, $R_{0,\text{max or min}}$ can be calculated according to

$$R_{0,\text{max or min}} = (1.5\langle \kappa^2 \rangle_{\text{max or min}})^{1/6} R_0(2/3) \quad (7)$$

By use of the maximum and minimum values of R_0 obtained in this way, maximum and minimum values of R can be calculated. The $^1/_6$th power dependence of R_0 on $\langle \kappa^2 \rangle$ greatly attenuates the effect of variation in the latter on the former.

Results

Homogeneity and Specificity of Labeling. The free SH groups generated by opening of the thiol esters of α_2M by either protease or methylamine have previously been labeled with high specificity by a number of thiol-specific reagents, such as DTNB and iodoacetamide derivatives. In a report examining the environment of the SH group, 1,5-I-AEDANS, the same reagent as used here to attach the donor fluorophore, was reacted with the four SH groups of protease- or methylamine-treated α_2M and found to be incorporated with the expected stoichiometry of 4 mol of dansyl per α_2M tetramer (Larsson et al., 1987), indicating efficient and selective reactivity with the SH groups. A study in which nitroxide spin-labels were attached to the thiol ester derived free SH groups, through reaction with iodoacetamido-PROXYL and iodoacetamido-TEMPO permitted an estimate of the degree of nonspecific labeling to be made, based on the very different spectral characteristics of the EPR signals from specifically and nonspecifically bound label (Gettins et al., 1988). A value of less than 2% was obtained.

I-Form α_2M was used in preparation of all of the fluorescent derivatives used in the present study. This form of α_2M has two free SH groups, derived from the two thiol esters in the first protease interaction site (Gettins et al., 1989). The observed stoichiometry of incorporation of fluorescent label of 2.0 ± 0.1 dansyl groups per α_2M tetramer, combined with the DTNB assay results, which showed no residual free SH groups after reaction with 1,5-I-AEDANS, and the earlier reports of highly specific reaction at these sulfhydryls strongly suggest that the dansyl groups are specifically incorporated at the desired SH groups in the first protease interaction site. There is also no evidence for a large proportion of nonspecific labeling from the fluorescence emission spectrum. Dansyl-labeled I-form α_2M shows a λ_{max} at 482 nm [cf. 483 nm reported previously for uniformly labeled α_2M (Larsson et al., 1987)], whereas *N*-acetylcysteine-AEDANS has a λ_{max} of 525 nm (Larsson et al., 1987). No shoulder corresponding to this red-shifted component is apparent in the labeled protein.

Similar considerations apply to the specificity of reaction of the second pair of thiol ester derived SH groups with 5-IAF. The much longer reaction times employed compared to 1,5-I-AEDANS (20 h vs 1 h) account for the slightly higher stoichiometries obtained (2.16–2.27 mol of label per α_2M tetramer). An earlier sample which had been reacted with 5-IAF for only 5 h, and which incorporated only 1.3 mol of acetamidofluorescein per α_2M tetramer, showed energy transfer in proportion to the fraction of specific sites occupied by acceptor. Also, the high efficiency of energy transfer, resulting from the close proximity of donor and acceptor (vide infra), makes the statistical likelihood of an appreciable additional contribution from transfer between donor and random nonspecifically linked acceptor unlikely in a molecule that has approximate dimensions of 90 × 190 Å (Feldman et al., 1985).

Characterization of Dansyl and Fluorescein Emission Spectra of Labeled α_2M. The spectral characteristics of (dansyl)$_2$-α_2M and (AF)$_2$-α_2M, prepared as described under Materials and Methods, are given in Table I. The dansyl-labeled species was prepared by reaction of the two SH groups of I-form α_2M with 1,5-I-AEDANS. Since generation of I-form α_2M through reaction with Sepharose-linked chymotrypsin leads to bait region and thiol ester cleavage in the first protease interaction site, but without trapping of protease, there is a difference between the dansylated half of the α_2M species reported here and the comparable half of the (dansyl)$_4$-α_2M

Table I: Absorption and Emission Properties of α_2M and Fluorescent Labels

sample	λ_{max} (nm)	μ_{max}(emission) (nm)	Φ_D
1,5-I-AEDANS[a]	342	525	0.02
$(dansyl)_2$-α_2M	342	482	0.26
$(dansyl)_4$-α_2M (protease treated)[a]	342	483	0.27
$(dansyl)_4$-α_2M (CH_3NH_2 treated)[a]	342	493	0.17
$(AF)_2$-α_2M	500	517	0.20
5-IAF[b]	490	514	0.89

[a] Taken from Larsson et al. (1987). [b] Taken from Seifried et al. (1988).

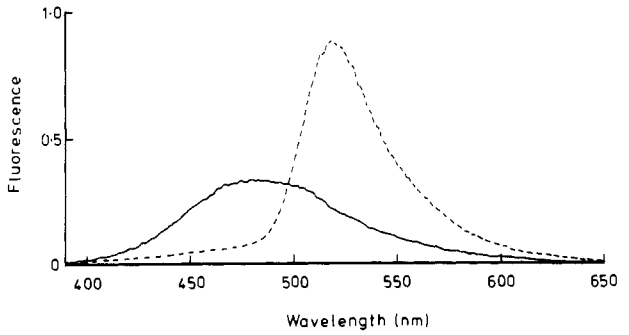

FIGURE 1: Fluorescence emission spectra of 0.5 μM $(dansyl)_2$-α_2M (solid line) and $(dansyl)_2$-$(AF)_2$-α_2M (dashed line) with excitation at 365 nm. Spectra were recorded at 298 K.

reported by Larsson et al. (1987) in that the latter has protease bound. It is therefore interesting to compare the properties of the dansyl fluorophore at equivalent sites in the two species. λ_{max} and Φ_D for the two are the same, despite significant differences in both parameters when comparing α_2M species labeled after opening of the thiol esters through protease reaction or direct cleavage with methylamine (Table I). The present findings thus emphasize that the differences seen in the latter two species reflect the consequences of bait region cleavage, rather than the presence of the protease, per se.

Fluorescent Properties of Dansyl/AF-Labeled α_2M. The fluorescence emission spectrum of $(dansyl)_2$-$(AF)_2$-α_2M is shown in Figure 1 together with the emission spectrum of an equal concentration of $(dansyl)_2$-α_2M. The doubly labeled protein shows a major emisssion peak due to fluorescein fluorescence but only a small amount of residual dansyl fluorescence, indicating a major quenching through fluorescence energy transfer from dansyl to fluorescein. To quantitate the decrease in dansyl and increase in fluorescein fluorescence intensity, it was necessary to correct the spectrum in Figure 1 for direct excitation of fluorescein at 365 nm. The difference spectrum obtained by subtraction of the spectrum of fluorescein-labeled α_2M from that of the doubly labeled protein is shown in Figure 2. This clearly has two components that correspond to the residual dansyl fluorescence (λ_{max} at 482 nm) and to the enhancement in fluorescein fluorescence through energy transfer rather than direct excitation. Superimposed on Figure 2 is the dansyl-α_2M fluorescence spectrum, reduced by 81.8%. The good fit at short wavelength between this spectrum and the spectrum of doubly labeled α_2M confirms that the origin of the shoulder in the latter is the dansyl groups and that the efficiency (E) of energy transfer is 81.8%. Upon subtraction of the scaled dansyl emission spectrum from the corrected doubly labeled emission spectrum, a spectrum is obtained that is well matched by that of fluorescein-labeled α_2M, directly excited at 365 nm (Figure 3). Quantitation of the increase in fluorescein emission was made by scaling of the residual in Figure 3 to the emission spectrum of fluorescein-labeled α_2M. An increase of 12.5%

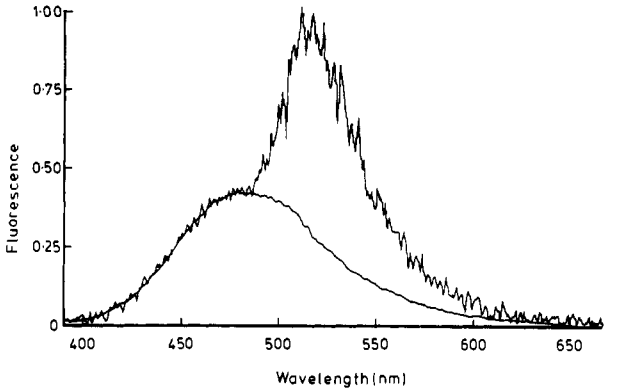

FIGURE 2: Fluorescence emission difference spectrum between $(dansyl)_2$-$(AF)_2$-α_2M and $(AF)_2$-α_2M at the same concentration (0.5 μM) (solid line). This difference spectrum represents the residual dansyl fluorescence of the doubly labeled sample together with the fluorescein fluorescence resulting from *resonance energy transfer* from the dansyl fluorophores. Superimposed on this difference spectrum is the emission spectrum of 0.5 μM $(dansyl)_2$-α_2M reduced by a factor of 5.48. This factor was chosen to give the best fit between the difference spectrum at short wavelength and the dansyl emission spectrum and thus gives a direct measure of the efficiency of transfer (E) as $(5.48 - 1)/5.48 = 81.8\%$.

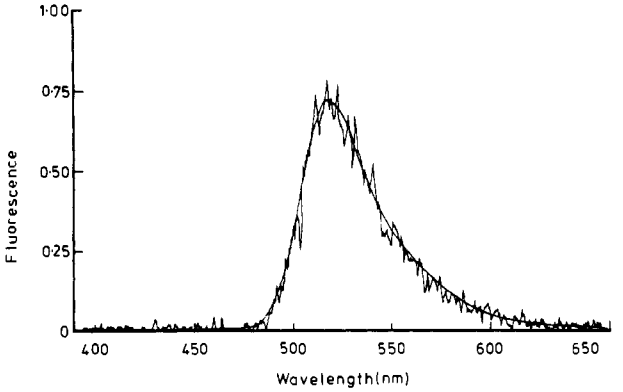

FIGURE 3: Fluorescence emission difference spectrum (light solid line) between the difference spectrum given in Figure 2 and the emission spectrum of $(AF)_2$-α_2M, where the AF groups are attached to the equivalent Cys949 SH groups as in the doubly labeled sample, i.e, in the second protease interaction site. This double difference spectrum represents the fluorescein emission enhancement and is matched well by the emission spectrum of 0.5 μM $(AF)_2$-α_2M reduced by a factor of 8.00.

in the fluorescein emission was found. This reflects the 81.8% energy transfer from danysl to fluorescein, which must then be multiplied by the quantum yield of the acceptor to yield the actual acceptor increase expected. This thus provides a means of estimating the acceptor's quantum yield. The value of 0.153 compares reasonably with the value of 0.198 determined directly and given in Table I.

Another way to demonstrate resonance energy transfer from donor to acceptor was to compare the excitation spectra of the doubly labeled sample and the two singly labeled control species. The excitation spectrum of doubly labeled α_2M monitored at 485 nm and corrected for the contribution from fluorescein alone is shown in Figure 4a together with the excitation spectrum of an equal concentration of $(dansyl)_2$-α_2M control. There is again a clear demonstration of a major loss in intensity in the region of dansyl excitation due to resonance energy transfer. In Figure 4b, the residual dansyl excitation is matched to the dansyl spectrum by multiplication using the scaling factor 3.6. As with the deconvoluted components shown in Figures 2 and 3, there is excellent superpositioning of the two spectra.

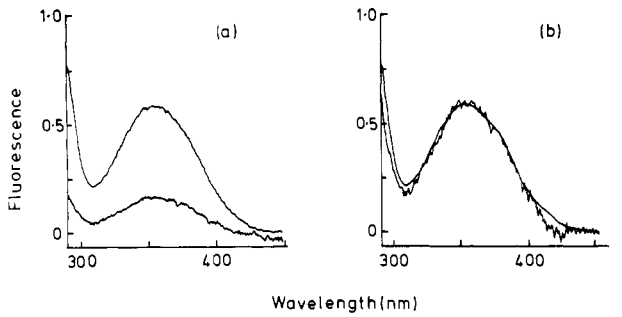

FIGURE 4: (a) Fluorescence excitation spectrum of $(dansyl)_2$-α_2M (top trace) and excitation difference spectrum of $(dansyl)_2$-$(AF)_2$-α_2M minus $(AF)_2$-α_2M (bottom trace). Fluorescence was monitored at 485 nm. Spectra were recorded at 298 K. (b) The same spectra as in (a), but with the difference spectrum multiplied by 3.6 to demonstrate the good fit between the two spectra.

Table II: Orientational Analysis of α_2M-Labeled Controls and Calculation of R_0 and R

	$\langle d_D \rangle$	$\langle d_A \rangle$	$\langle d_{D^x} \rangle$	$\langle d_{A^x} \rangle$	$\langle \kappa^2 \rangle$	R_0 (Å)	R (Å)
isotropic					0.667	43.5	33.9
max	0.768	0.483	0.876	0.694	2.930	55.7	43.4
min	0.768	0.483	0.876	0.694	0.136	33.5	26.1

Calculation of κ^2 and R_0. Since J, the spectral overlap integral, and Φ_D, the quantum yield of the donor, can be directly determined experimentally, and n, the refractive index of the medium between the donor and acceptor fluorophores, is usually taken to be 1.4 for protein systems (Beardsley & Cantor, 1970), the major uncertainty in calculating R_0 and therefore the separation, R, between the fluorophores is in choosing a value for κ^2, the orientation factor. To obtain maximum and minimum values of κ^2, and thus maximum and minimum values of R_0, fluorescence anisotropy measurements were performed on both donor only and acceptor only protein samples, as described under Materials and Methods. Values of 0.307 and 0.193 were obtained for the donor and acceptor, respectively. The resulting values of $\langle \kappa^2 \rangle_{max\ or\ min}$ were calculated according to eq 3–6 and are given in Table II. A value of $R(2/3)$ of 33.9 Å was calculated by using the value of 1.7×10^{-13} M^{-1} cm^3 calculated for J and a quantum yield for the donor of 0.26 (Table I). The values for $R_{max\ and\ min}$ calculated by using the maximum and minimum values of κ^2 and eq 7, are 43.4 and 26.1 Å, respectively.

Discussion

The results presented here are the first realization of the usefulness of I-form α_2M in helping to answer questions concerning the structure and organization of α_2M. This form of α_2M has two free SH groups in the first protease binding site and intact thiol esters in the second. I-Form can then react with and trap protease in the second binding site and as a result generate two new SH groups from the pair of thiol esters in the second site. This ability to liberate SH groups from the two thiol esters in the first protease interaction site, independently from those in the second site, has enabled specific incorporation of different fluorophores to be achieved at the two pairs of SH groups. By appropriate choice of fluorophores, fluorescence resonance energy transfer measurements have been possible. By good fortune, the separation between donor and acceptor is in the region where the efficiency of transfer is sensitive to distance, being neither so close that transfer is indistinguishable from 100% nor so far that transfer is close to 0%. Furthermore, the anisotropy values determined for both donor and acceptor are small enough that the uncertainty in separation, determined as the spread between R_{max} and R_{min}, is modest.

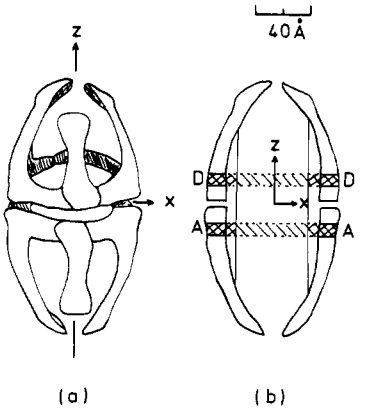

FIGURE 5: (a) Model of α_2M based on Feldman et al. (1985) and modified slightly by removal of the proteases (which would be located in the upper and lower cavities in a 2:1 protease complex). The axes are those referred to in the text. This model is based in large part on electron microscopy data and shows a hollow molecule with annuli of protein density in contact in the center of the molecule with two types of arms of protein density attached to these rings and extending outward along the (+) and $-z$ directions to form the "walls" of the protease trap. (b) An arbitrary vertical slice through the structure shown in (a) and corresponding to the xz plane. The separation between the pairs of horizontal lines drawn above and below the xy plane represents the upper and lower limits of R calculated from the resonance energy transfer experiments presented here, assuming that the four Cys949 residues lie in a plane that also contains the z axis. The hatched areas between pairs of lines represent possible locations for the cysteine residues with these constraints. Of course, much of this area does not intersect protein density and would be considered improbable on this ground alone. The vertical lines, symmetrically placed about the z axis, represent further limits imposed by other experiments referenced in the text. Since the Cys residues must lie further from the z axis than these lines, the areas available to the cysteine residues are only those that are cross-hatched. Most of these areas do intersect protein density and correspond to the locations predicted by Feldman et al. (1985).

Localization of the Cys949 Residues in Reacted α_2M. The determination of the separation between Cys949 residues in different protease binding sites of α_2M has important consequences for the localization of these residues within the α_2M tetramer. Figure 5a reproduces the hypothetical structure of reacted α_2M proposed by Feldman et al. (1985) on the basis of then available physical and chemical data. In overall features, it still seems to be compatible with more recent immunoelectron microscopy studies (Delain et al., 1988) and so will be used here as a basis for interpretation of the present data. An important feature of the model is its symmetry. The α_2M tetramer is a dimer of noncovalently associated disulfide-linked pairs of subunits. While there is still uncertainty as to which plane in Figure 5a represents the face of noncovalent interactions, i.e., xy, xz, or yz, there is general agreement that the reacted form α_2M has high, possibly D_2, symmetry. Data in support of this come from such diverse sources as immunoelectron microscopy (Delain et al., 1988), rates of reaction of the thiol esters with methylamine (Strickland & Bhattacharya, 1984), the equivalence of ^{1}H NMR signals from the four bait regions in both native and methylamine-treated α_2M (Gettins, & Cunningham, 1986b), and the stoichiometry of high-affinity zinc binding sites on α_2M (Gettins & Cunningham, 1986a). Given this model for α_2M, what limitations do the current data impose upon possible locations for Cys949 residues? Since a major part of the question of location is how far out from the central disk in the z direction the residues lie, consideration will first be given to this question. The maximum separation above or below the xy plane (Figure 5a) for these residues will occur if all four are coplanar (see below). Consider an arbitrarily chosen slice that contains the z axis

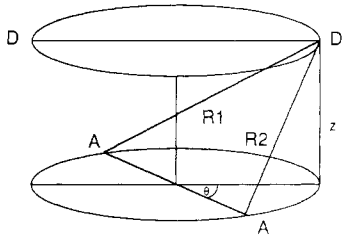

FIGURE 6: Representation of the general case of pairs of donors and acceptors in parallel xy planes that are rotated about z with respect to one another by θ. The donor–acceptor separations are R_1 and R_2. Because of C_2 symmetry about the z axis, each donor is equivalent.

(Figure 5b). The hatched regions correspond to separations for a donor/acceptor pair that are within the range of values delimited by R_{max} and R_{min}.

Further localization in the x direction comes from other studies. Thus, it has been observed that when covalent cross-links are formed between the thiol ester derived Glu residues of both thiol esters and lysine side chains on the trapped protease, the two lysine residues are located on opposite sides of the molecule (Sottrup-Jensen, 1987). This suggests a separation of thiol esters *within* a protease binding site equal to the diameter of the protease plus approximately 10–15 Å to account for the ϵ-amido linkages. This gives a value of 50–55 Å. Such a value is also consistent with EPR measurements which showed no dipole–dipole interaction between nitroxide spin-labels attached to the four Cys949 residues, giving a minimum pairwise separation of the nitroxides of 20 Å and a likely separation of the SH groups to which they are attached of more than 38 Å, again *within* a protease binding site, when the length and probable relative orientations of the linker arms are taken into account (Gettins et al., 1988). Another EPR study of chymotrypsin noncovalently complexes to α_2M demonstrated that the protease was free to rotate in the interior of the inhibitor (Crews et al., 1987) and thus imposed minimum dimensions on the size of the cavity of approximately 50-Å diameter. Restricting the two thiol ester residues, within a single trapping unit, to an x separation of >50 Å further limits the area available for these residues to the cross-hatched area of Figure 5b. This intersects a region of protein density corresponding to one of the arms of the molecule in the arbitrarily chosen vertical section.

It should be remembered that the section chosen above was arbitrary and also that the simplest case of coplanar thiol groups was considered. Furthermore, the effect of energy transfer to the second acceptor was ignored. For the separation between pairs of donors or acceptors of 50 Å suggested above, the effect on the shorter donor/acceptor separation of including the contribution of transfer to the second acceptor of 3% is insignificant, because of the sixth root dependence of distance on transfer efficiency. Now consider the consequences of the four SH groups not being coplanar, though with the xy plane containing the pair of donors still being parallel to the plane containing the pair of acceptors (Figure 6). θ represents the angle through which the pair of acceptors have been rotated about the z axis from coplanarity, and z represents the separation between the xy plane containing the pair of donors and the xy plane containing the pair of acceptors. The limitations imposed on the two donor/acceptor separations (R_1 and R_2) by this symmetry and the requirement to produce the observed efficiency of energy transfer, E, lead to the following relationship between θ and z:

$$\left(D^2 \sin^2 \frac{\theta}{2} + z^2\right)^{-3} + \left(D^2 \cos^2 \frac{\theta}{2} + z^2\right)^{-3} = \frac{E}{(1 - E)R_0^6} \quad (8)$$

where D is the separation between donor pairs or acceptor pairs. This function has a maximum value for z at $\theta = 0, 180°$, which corresponds to the coplanar arrangement of all four fluorophores that was considered above, and a minimum value at $\theta = 90°, 270°$, which corresponds to each acceptor being midway from each donor. The values of θ that correspond to these maxima and minima are independent of both D and E. Thus, any geometry other than coplanar would bring the SH groups even closer to the central xy plane. The minimum value of z, for $\theta = 90°$, is 13.7 Å using $R_0(2/3) = 43.5$ Å and $D = 50$ Å. While the present data do not allow a conclusion to be reached as to the positions of the four Cys residues projected onto the xy plane, it is clear that these residues must be within the central region of the molecule, maximally 20 Å above and below the central plane, but possibly even closer if they do not lie directly beneath one another. This conclusion is opposite to that reached by Schramm and Witke using electron microscopic visualization of ferritin–avidin–biotin–α_2M complexes (Schramm & Witke, 1988) but is exactly the prediction made by Feldman et al. (1985) based on a location that would not only allow close access to the protease, as required by fluorescence energy transfer studies of Pochon et al. (1983), but also more readily explain the extent of the conformational change that results from thiol ester cleavage. The central location required by the present studies is also attractive from the point of view of being able to accomodate a number of the properties of α_2M. Thus, since the bait region is close to the thiol ester (Gettins et al., 1988), a central location for the bait regions could account for the slow cleavage of the remaining pair of bait regions by protease in binary complex with α_2M (Roche & Pizzo, 1987; Strickland et al., 1988), if there is indeed a wide solvent channel connecting the two protease binding sites. Such a channel has been suggested from low-angle, low-resolution, X-ray scattering studies on α_2M in solution (Österberg & Pap, 1983). In addition, the high efficiency of trapping of proteases that cleave bait regions suggests that the proteases are well positioned for sequestration at the time of proteolysis. This is more consistent with bait regions placed well toward the center of the α_2M interior than closer to the ends of the arms.

SUMMARY

Fluorescence energy transfer measurements on fluorophores attached to the SH groups derived from the thiol esters of α_2M have shown that the thiol esters in different protease interaction sites are located no more than 35.2 ± 8.6 Å apart. Further lateral restrictions can be imposed on the basis of other published data. Together this restricts the thiol esters, and in turn the bait regions, to the central disk region of the tetramer visualized by electron microscopy. This localization is in harmony with the known properties of α_2M.

Registry No. Cys, 52-90-4.

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