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α_2-Macroglobulin bait region integrity

Role in determining fast-form structure

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To determine whether integrity of the bait region affects the structure of the remainder of human α_2-macroglobulin (α_2M), we have determined the separation between cysteine residues in a methylamine-reacted fast-form of α_2M. From reduction in fluorescence intensity of covalently-bound donor fluorophore caused by proximity to an acceptor, a separation of 35 ± 8 Å was calculated, which is identical to a previously determined value for proteinase-treated fast-form α_2M. This indicates that although bait region cleavage is the physiological route to conformational change in α_2M, bait region integrity per se does not significantly affect the structure of fast-form α_2M.

α_2-Macroglobulin; Bait region; Fluorescence resonance energy transfer

1. INTRODUCTION

The inhibition of proteinases by α_2-macroglobulin (α_2M) involves a large-scale conformational rearrangement of the inhibitor that is initiated by cleavage of the α_2M bait region by the proteinase. Bait region cleavage in turn makes the internal thiol ester, present between Cys^{949} and Glx^{952} of each of the identical subunits of tetrameric α_2M, more susceptible to nucleophilic attack and thus to cleavage, which results in the conformational changes in α_2M that trap the proteinase [1].

It has been assumed that the α_2M species produced by cleavage of the thiol ester by methylamine, in the absence of proteinase, has the same overall structure as the species produced by reaction with proteinase. This assumption is based on similarities in properties of the two species, including UV [2] and CD [2,3] spectra, mobility on non-denaturing polyacrylamide gels [4], and sedimentation coefficients [2]. These, however, are aggregate properties that cannot be interpreted directly in structural terms. A more direct electron microscopy image reconstruction study indicated that the two α_2M species have similar structures [5]. This comparison is, however, limited by the low resolution of the method, which cannot distinguish differences of less than 30 Å.

We report here the results of distance measurements between the Cys^{949} residues in an α_2M in which the thiol esters in the second half of the molecule have been cleaved by methylamine (Fig. 1) to answer the question of what effect the integrity of the bait region has on the structure of the protein. A separation indistinguishable from one found previously for a proteinase-treated fast-form α_2M [6] was calculated from fluorescence resonance energy transfer measurements, indicating very similar structures for the two species and thus no detectable effect of bait region integrity on the overall conformation of the protein.

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Abbreviations: α_2M, α_2-macroglobulin; I-form α_2M, intermediate form α_2M; PAGE, polyacrylamide gel electrophoresis; 5-IAF, 5-iodoacetamidofluorescein.

2. MATERIALS AND METHODS

2.1. *Preparation of α_2M species*

Human α_2M was purified from recently expired plasma as previously described [7]. The material was free from contaminants, as judged by PAGE under both denaturing and non-denaturing conditions. PAGE was performed in 5% acrylamide slabs. α_2M concentrations were determined spectrophotometrically using $A^{1\%}_{280\,nm} = 8.9$ [7] and a molecular weight of 716 kDa [8]. I-form α_2M was prepared as previously described [9] by reaction of native α_2M with chymotrypsin-Sepharose.

2.2. *Preparation of fluorescent α_2M derivatives*

α_2M species, labeled at Cys^{949} residues with either dansyl or fluorescein groups, were prepared using methods previously described [6]. Specific attachment of dansyl to the two cysteines in the first half of α_2M and of fluorescein to the two cysteines in the second half of the protein involved initial reaction of I-form α_2M with 1,5-IAEDANS, followed by opening of the second pair of thiol esters with methylamine followed by reaction with iodoacetamidofluorescein. Reaction conditions for the labeling have been described previously [6].

2.3. *Nomenclature for α_2M derivatives*

All α_2M species examined in this study carried labels on each of the four Cys^{949} residues. These labels were either fluorescein, dansyl, or

acetamide. Abbreviations used to indicate label composition are F, D, and aa respectively. The order indicates in which half of the α_2M the label is located. The use of (M) or (P) after a species signifies that methylamine or proteinase, respectively, was used to open the second pair of thiol esters. Thus D_2F_2(M) represents α_2M labeled with two dansyl groups in the first half of the molecule, and two fluorescein labels in the second half, with methylamine used to cleave the second pair of thiol esters.

2.4. *Fluorescence measurements*

Steady state fluorescence emission spectra were recorded on an ISS Greg 200 fluorometer operated in analog mode. An excitation/emission bandwidth of 6 nm was used. 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. Values for κ^2 were calculated from anisotropy measurements of the dansyl and fluorescein groups in D_2aa(M) and aa_2F_2(M) respectively, as described [6].

2.5. *Calculation of* R_0 *and separation between donor–acceptor pair*

R_0, the separation for 50% efficiency of transfer was calculated from the relationship:

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

where J is the spectral overlap integral, κ^2 is the orientation factor, and Φ_D is the quantum yield of the donor. These values were calculated for the present system as described [6]. A value of 1.4 was used for the refractive index (n).

2.6. *Materials*

Chymotrypsin–Sepharose was prepared as described [9]. 1,5-IAEDANS was from Aldrich Chemical Co., 5-IAF was from Molecular Probes, Eugene, Oregon, and DTNB was from Sigma Chemical Co.

3. RESULTS

3.1. *Fluorescence resonance energy transfer in* D_2F_2*(M)* α_2*M*

From a comparison of the fluorescence emission spectra of D_2F_2(M) and D_2aa_2(M) (Fig. 2) it can be seen that introduction of the fluorescein acceptor fluorophore in the second half of the α_2M molecule resulted in a large reduction in the dansyl emission intensity centered at 482 nm. To quantitate the reduction, the emission spectrum of the D_2F_2(M) was corrected for any contribution from direct excitation of fluorescein at 365 nm by subtracting the emission spectrum of aa_2F_2(M). The resulting difference spectrum (Fig. 3) represents the residual dansyl fluorescence, together with the enhancement of fluorescein fluorescence resulting from resonance energy transfer between dansyl and fluorescein. Comparison of the intensity of the difference spectrum in the region 460 to 470 nm, i.e. close to the dansyl emission maximum but in a region where fluorescein does not contribute, with the emission spectrum of D_2aa_2(M) showed an 83% reduction in the intensity of dansyl fluorescence resulting from energy transfer to fluorescein in the doubly labeled species D_2F_2(M).

An independent way of demonstrating resonance energy transfer from donor to acceptor was to compare the excitation spectrum of D_2F_2(M), monitored close to the dansyl emission maximum and corrected for any contribution of the fluorescein alone by subtraction of the corresponding aa_2F_2(M) excitation spectrum, with that of D_2aa_2(M). The same large reduction in intensity (83%) was found (Fig. 4) as for the reduction in the fluorescence emission spectrum (Fig. 2).

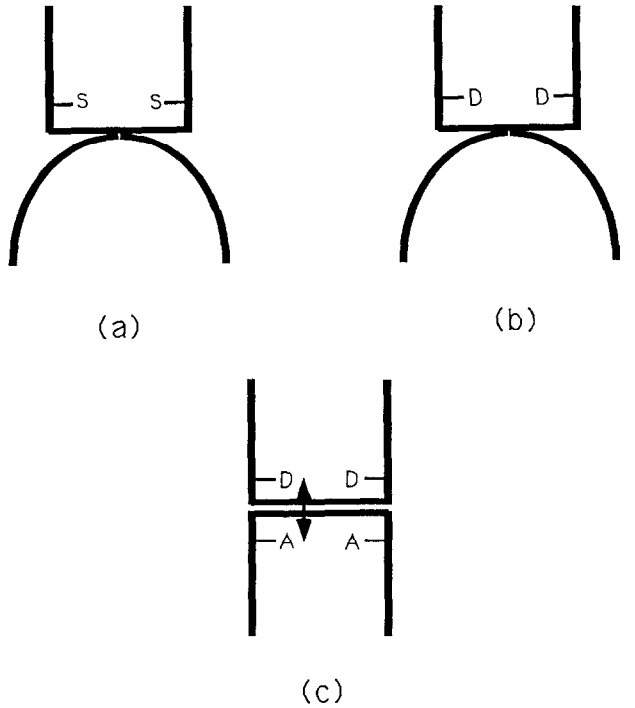

Fig. 1. Schematic representation of α_2M species used in this study, showing location of Cys^{949} residues and the inter-fluorophore separation measured. Half molecules of α_2M are shown as curved or rectilinear to represent native or altered conformations, respectively; this is schematic and not meant to depict the actual shape of these units. (a) I-form α_2M showing the two free Cys^{949} side chains in the first half of the molecule. (b) Dansyl labeled I-form α_2M, which is the starting point for subsequent generation of both D_2F_2(M) and D_2F_2(P). (c) D_2F_2(M) or D_2F_2(P), showing the location of the donor and acceptor fluorophores and the separation measured by fluorescence resonance energy transfer (arrow); the difference between D_2F_2(M) and D_2F_2(P) is in the use of methylamine or proteinase respectively to generate the free Cys^{949} side chains in the **second** half of the α_2M.

3.2. *Separation between donor and acceptor fluorophores*

Using the calculated values of R_0 and κ^2 (Table I), and the observed reduction of 83% in dansyl fluorescence due to resonance energy transfer, a separation between donor and acceptor fluorophores in D_2F_2(M) of 34.8 ± 8.8 Å was calculated. This compares with a value of 34.8 ± 8.6 Å calculated previously for D_2F_2(P) [6]. It should be noted that the values given represent the separation calculated for a κ^2 value of 2/3 (isotropic rotation of fluorophores) and the maximum and minimum separations derived from the maximum and minimum values of κ^2 consistent with the observed fluores-

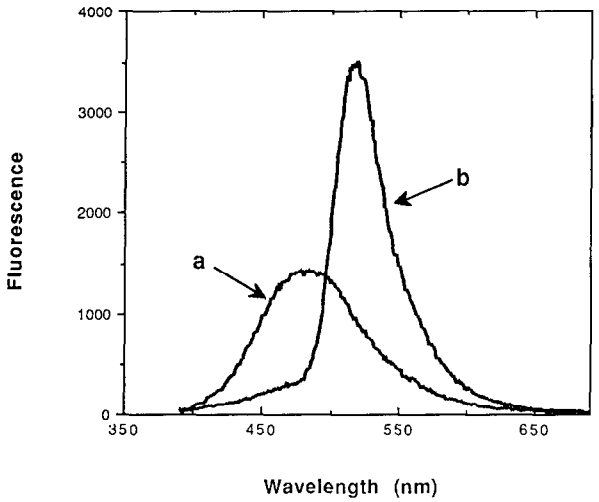

Fig. 2. Fluorescence emission spectra of (a) D_2aa_2(M) and (b) D_2F_2(M) recorded at equal concentrations of 0.3 μM.

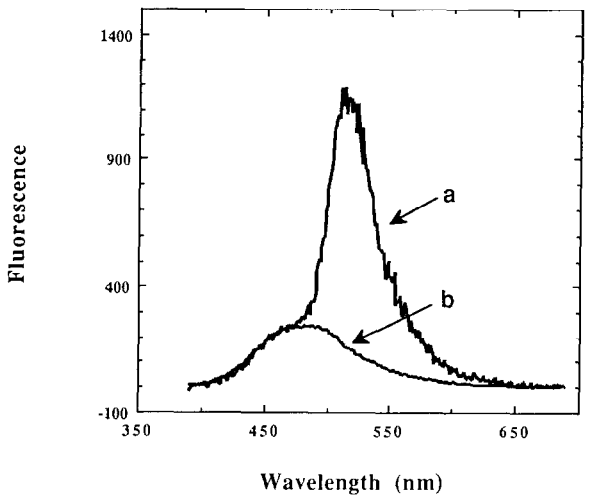

Fig. 3. (a) Fluorescence emission difference spectrum between D_2F_2(M) and aa_2F_2(M) at the same concentration (0.3 μM). The difference spectrum represents the residual dansyl fluorescence of the doubly labelled sample as well as the contribution to fluorescein fluorescence resulting from resonance energy transfer from the dansyl fluorophores. (b) Emission spectrum of D_2aa_2(M) reduced by 83% is superimposed and shows an excellent fit to the difference spectrum in the region where only dansyl fluorescence contributes. The scaling factor gives a direct measure of the efficiency of transfer (E).

cence anisotropies. Since the anisotropies for D_2F_2(M) and D_2F_2(P) differ only slightly (Table I), the uncertainty in the *difference* in separations between the fluorophores in the two forms of α_2M (i.e. separation in D_2F_2(M) minius separation D_2F_2(P)) is likely to be much less than the uncertainty in the separation itself.

4. DISCUSSION

We have shown in the present study that bait region cleavage per se does not alter the location of the Cys^{949} residue in that subunit of α_2M within experimental error. Together with the overall similarity of structure at low resolution between proteinase- and methylamine-treated α_2M shown by electron microscopy [5], this indicates that the two forms of α_2M are structurally equivalent, with the exception of the cleavage of bait regions and presence of trapped proteinase in the former.

The approach used, of starting with I-form α_2M to permit specific attachment of donor fluorophore in the first half of the molecule, with subsequent labeling of the second half with acceptor fluorophore following opening of the thiol ester with either proteinase or methylamine, has the advantage that the location of the donor fluorophores in both D_2F_2(M) and D_2F_2(P) is the same, so that comparison of the two structures should reveal differences arising only from a change in position of the acceptor fluorophore.

Although a relatively large range of values is given for the possible separation of donor and acceptor fluorophore within a given α_2M species (Table I), resulting from the uncertainty in the orientation factor (κ^2), the uncertainty in the difference in separation of the fluorophores between D_2F_2(M) and D_2F_2(P) is likely to be very much less than this. This is because of the similarity of the anisotropies for D_2F_2(M) and D_2F_2(P), from which the maximum and minimum values of κ^2 were calculated. Although there is large uncertainty in κ^2 for each species, the actual value is likely to be very similar for each form. The uncertainty in the *difference* in separation for the two α_2M species would then be largely determined by the uncertainty in the efficiency of energy transfer. We estimate, from the observed variation in the measured efficiency of resonance energy transfer, that this can be determined to ± 3%. This corresponds to an uncertainty in the separation of less than 1 Å, due to the r^{-6} dependence of the efficiency of energy trans-

Table I

Energy transfer parameters for fluorescently-labeled α_2M species

	R_0(2/3) (Å)	R_0max (Å)	R_0min (Å)	E (%)	R (Å)	A_{donor}	$A_{acceptor}$
D_2F_2(M)	44.1	56.6	33.8	83	35 ± 9	0.290	0.220
D_2F_2(P)	43.5	55.7	33.5	82	35 ± 9	0.307	0.193

Anisotropies were determined as described in [6] and used to calculate maximum and minimum values of R_0 as described [6]. Data for D_2F_2(P) were taken from [6].

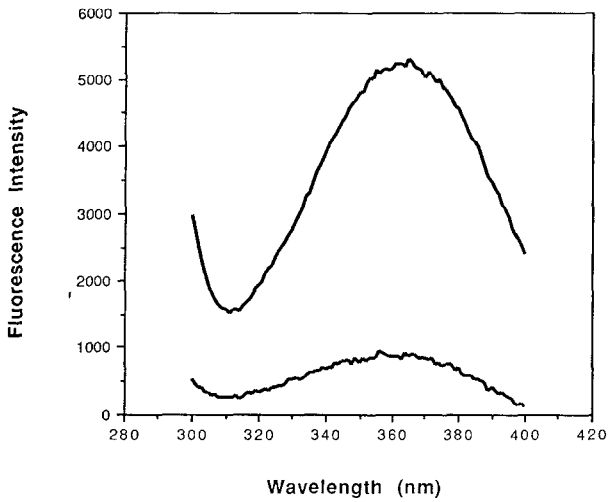

Fig. 4. Fluorescence excitation spectrum of $D_2aa_2(M)$ (top trace) and excitation difference spectrum of $D_2F_2(M)$ minus $aa_2F_2(M)$ (bottom trace). The scaling factor necessary to bring about correspondence of the two spectra in the region of the dansyl excitation maximum is 5.9. This corresponds to an 83% reduction in dansyl excitation intensity in $D_2F_2(M)$ resulting from fluorescence resonance energy transfer.

fer. This resolution is much better than that obtained by image reconstruction electron microscopy [5], though the latter has the advantage that it reports on the whole structure. Using the latter result to provide the grossly similar outline of both forms of α_2M and the present finding of indistinguishable locations for the Cys^{949} residues in each form, we conclude that the two α_2M fast-form species are structurally equivalent.

This similarity implies that the bait region interacts minimally with the remainder of α_2M. This is consistent with the exposed, flexible nature of this region [10,11] and the ability of α_2Ms from different species to accommodate bait regions of very different lengths and compositions. A plausible mechanism for bait-region-cleavage-mediated change in α_2M conformation is that the bait region is a discrete surface domain positioned close to the thiol ester [12] in a way to restrict access by solvent or other nucleophiles. Cleavage of the bait region at any site within it removes this restriction and permits opening of the thiol ester and consequently the trapping conformational change.

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