Volume 332, number 3, 211–214 FEBS 13098 October 1993

Human α_2-macroglobulin structure

Location of Cys-949 residues within a half-molecule measured by fluorescence energy transfer

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Received 23 July 1993

To localize the pair of thiol-ester-derived cysteine-949 residues within a half-molecule of α_2M and estimate the internal diameter of this α_2-macroglobulin trap, we have measured the separation between these cysteines. We unexpectedly found that the two cysteines of intermediate form α_2M had different reactivity, which permitted selective modification of one cysteine with dansyl, and the other with fluorescein fluorophores. From fluorescence energy transfer measurements, we calculated a separation of 41 ± 10 Å between these fluorophores. This indicates that the Cys-949 residues are probably located at the perimeter of the trap with an internal diameter at least as large as this separation.

α_2-Macroglobulin; Fluorescence resonance energy transfer; Thiol ester; Structure

1. INTRODUCTION

Determination of the location and environment of the bait region and thiol esters within the α_2-macroglobulin (α_2M) tetramer is a major goal in studies aimed at explaining the mechanism, efficiency, and specificity of proteinase inhibition by α_2M. In an earlier study [1] we used donor and acceptor fluorophores, covalently attached to the four Cys-949 side chains that are involved in thiol ester formation, to show that the thiol groups are centrally positioned in the H-shaped molecule, with a longitudinal separation of 35 ± 9 Å (Fig. 1a). In that study the symmetric distribution of donor fluorophores in one half of the α_2M molecule and acceptors in the second half did not permit measurement of the transverse separation between pairs of Cys-949 residues. To determine that separation, which represents the minimum diameter of the internal cavity of the α_2M, we have specifically modified two Cys-949 thiols within one half of α_2M with a donor/acceptor fluorophore pair (Fig. 1b) and measured the efficiency of resonance energy transfer between the two fluorophores. Such specific modification was possible because of previously unsuspected differential reactivity of the two SH groups in intermediate form (I-form) α_2M. We report here the results of these studies, which showed that the Cys-949 residues within a half molecule of α_2M are 41 ± 10 Å apart.

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Abbreviations: α_2M, α_2-macroglobulin; I-form α_2M, intermediate form α_2-macroglobulin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; 5-IAF, 5-iodoacetamidofluorescein; 1,5-I-AEDANS, 5[[2-[(iodoacetyl)amino]-ethane]amino]naphthalene-1-sulfonate. PMSF, phenylmethanesulfonyl fluoride.

2. MATERIALS AND METHODS

2.1. *Purification of α_2-macroglobulin*

Human α_2M was purified from outdated plasma (Vanderbilt Blood Bank) by chromatography on zinc-chelate resin and gel chromatography as previously described [2]. Purity was judged to be >95% by polyacrylamide gel electrophoresis under both native [3] and denaturing conditions [4]. α_2M concentrations were determined spectrophotometrically using $E^{1\%}_{280nm} = 8.9$ [5] and a molecular weight of 716 kDa based on the sequence and carbohydrate content [6].

2.2. *Preparation of intermediate-form α_2-macroglobulin*

Intermediate-form α_2M was prepared by reaction of native α_2M with Sepharose-immobilized chymotrypsin as previously described [7]. The progress of the reaction was monitored by DTNB assay of the concentration of free SH groups in the α_2M. Reaction was terminated by centrifugation and filtration of the supernatant through an 0.2 μm filter. The recovered I-form α_2M had the expected [7] intermediate mobility on non-denaturing polyacrylamide gels, contained 2 free SH groups (1.9 ± 0.1), 2 intact thiol esters, which could be subsequently cleaved with methylamine (release of additional 1.7 ± 0.1 SH groups), and had half cleaved and half intact bait regions, judged from SDS-PAGE.

2.3. *Preparation of fluorescent derivatives*

Fluorescent covalent derivatives of I-form α_2M were prepared by reaction of free SH-containing samples with iodoacetamido-containing reagents, either 5-IAF or 1,5-I-AEDANS. Incorporation of dansyl fluorophore was estimated both from measurement of the absorption at 342 nm, using an extinction coefficient of 6.3×10^3 $M^{-1} \cdot cm^{-1}$ [8], and from the fluorescence intensity relative to that of I-form α_2M

labeled at both SH groups with a large excess of 1,5-I-AEDANS. Details of the reaction with 1,5-I-AEDANS are given under Results. Reaction with 5-IAF was carried out at 4°C in the dark with a 20-fold molar excess of reagent [9]. The stoichiometry of fluorescein incorporation was determined from the absorbance at 494 nm using an extinction coefficient of 7.13×10^4 $M^{-1} \cdot cm^{-1}$ [9].

2.4. *Fluorescence measurements*

Steady-state fluorescence emission and excitation spectra were recorded on an SLM8000 spectrofluorometer. Excitation and emission bandwidths of 4 nm were used. R_0 (the separation for 50% efficiency of transfer) was calculated using the relationship [10]:

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

J is the spectral overlap integral, κ^2 is the orientation factor, Φ_D is the quantum yield of the donor, and n is the refractive index of the medium, taken as 1.4 [11]. Maximum and minimum values of the orientation factor were determined from anisotropy measurements of donor and acceptor fluorophores [10,12]. The dansyl quantum yield was determined by reference to the fluorescence intensity of quinine sulfate in 50 mM sulfuric acid [10]. The separation, R, between donor and acceptor fluorophores was calculated using the relationship:

$$R = R_0(1/E - 1)^{1/6} \quad (2)$$

where E is the efficiency of energy transfer, with use of the appropriate value for R_0 depending on whether the maximum, minimum, or isotropic value for κ^2 was used.

2.5. *DTNB assay*

The concentration of free sulfhydryl groups was determined spectrophotometrically by quantitating release of thionitrobenzoate from DTNB, monitoring the change in absorbance at 410 nm [13].

2.6. *Materials*

Chymotrypsin-Sepharose was prepared as described [7]. 1,5-I-AEDANS was from Aldrich Chemical Co., 5-IAF was from Molecular Probes, Eugene, OR, and DTNB was from Sigma Chemical Co.

3. RESULTS

3.1. *Differential reactivity of SH groups in I-form α_2M*

In previous fluorescence energy transfer experiments on α_2M [1,14], both SH groups of a pair within a proteinase binding site were labeled with the same fluorophore (Fig. 1a). A molar excess of labeling reagent could therefore be used. In the present study we wanted to attach two different fluorophores to the pair of SH groups present in the reacted half of I-form α_2M (Fig. 1b). Although we expected no difference in reactivity of the two SH groups towards a given labeling reagent, and therefore a statistical distribution of incorporated label, we examined the kinetics of reaction of I-form α_2M with 1,5-I-AEDANS and with DTNB to determine if each SH group did react equivalently. Surprisingly, we found significantly different rates of reaction of the two SH groups of I-form α_2M with both 1,5-I-AEDANS and DTNB. Much faster incorporation of the first dansyl label was found than of the second (Fig. 2) when I-form α_2M was reacted with four equivalents of 1,5-I-AEDANS. The first phase was complete within 15 min under the conditions used. Reaction of the second SH group was at least four times slower. When a large excess 1,5-I-AEDANS was used for the reaction, the rate of each phase was increased by an amount consistent with a bimolecular rate limiting step (data not shown). However, the maximum incorporation of AEDANS was independent of the molar excess of reagent, and corresponded to 2 mol AEDANS/mol α_2M.

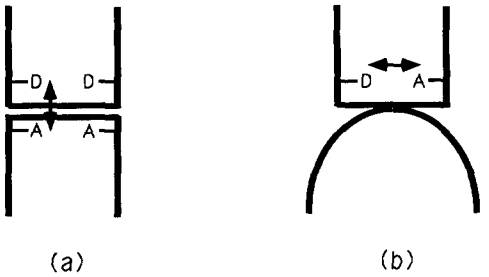

Fig. 1. Schematic representations of the α_2M tetramer, showing the type and location of fluorophores and the inter-cysteine separation measured in (a) previous studies [1,15], and (b) the present study. Half molecules of α_2M are shown as curved or rectilinear to represent native or reacted conformations. This depiction is schematic and is not meant to represent the actual shape of these units. Also, no attempt is made to distinguish between the possibilities that a half molecule is composed of two monomers from the same disulfide-bonded 360 kDa dimer or from monomers from separate 360 kDa dimers. **D** represents a dansyl (donor) fluorophore, and **A** represents a fluorescein (acceptor) fluorophore.

The reaction of I-form α_2M with DTNB was much more rapid than with 1,5-I-AEDANS, but still showed biphasic kinetics. Using a much lower concentration of DTNB than normally used in a DTNB assay, and corresponding to 2 equivalents of DTNB per α_2M, an initial burst of thionitrobenzoate release was observed in the first few seconds, corresponding to modification of about 1.0 mol SH/α_2M, and was followed by slower modification of the second SH group (data not shown).

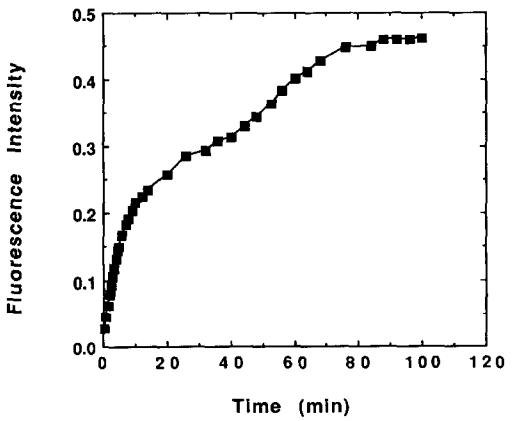

Fig. 2. Time course of the reaction of 1,5-I-AEDANS with I-form α_2M, monitored by change in dansyl fluorescence at 480 nm with excitation at 365 nm. 2.1 μM I-form α_2M was reacted with four equivalents of 1,5-I-AEDANS at 25°C in 0.05 M HEPES buffer pH 7.5 containing 0.1 M NaCl and 2 mM EDTA. These are the same reaction conditions used for the preparative dansyl-labeling of I-form α_2M. The solid line is for visual aid only.

The total thiobenzoate release at 20 min approximated that observed with the same sample reacted with the normal large excess of DTNB (0.1 mM); 1.5 mol versus 1.8 mol/α_2M, respectively.

3.2. *Preparation and characterization of dansyl/fluorescein-labeled I-form α_2M*

We exploited this differential reactivity of the two Cys-949 SH groups in I-form α_2M to preferentially modify one SH group with donor (dansyl) fluorophore and the second with acceptor (fluorescein) fluorophore (Fig. 1b). I-form α_2M was reacted with 1.0 eq 1,5-I-AEDANS for 1 h at room temperature; conditions under which it was expected that all of the 1,5-I-AEDANS should have reacted. The sample was divided into four aliquots. Three parts were incubated with a 20-fold molar excess of 5-IAF for 6, 18, or 42 h in the dark, while the fourth part was reacted with 10 mM iodoacetamide to block remaining free SH groups. For the samples reacted with 5-IAF for 6, 18, and 42 h, the stoichiometries of label incorporation were 0.51, 0.63 and 0.83, respectively. Quantitation of dansyl incorporation was made on the aliquot treated with iodoacetamide and gave a value of 1.0 per tetramer.

3.3. *Fluorescence resonance energy transfer in dansyl/fluorescein-labeled I-form α_2M*

The effect of introduction of a fluorescein acceptor fluorophore into dansyl-labeled I-form α_2M was to greatly reduce the intensity of the dansyl emission (Fig. 3). The reduction in dansyl emission intensity for labeled α_2M samples containing 0.51, 0.63, and 0.83 fluorescein labels/α_2M tetramer (Fig. 3b–d), but identical

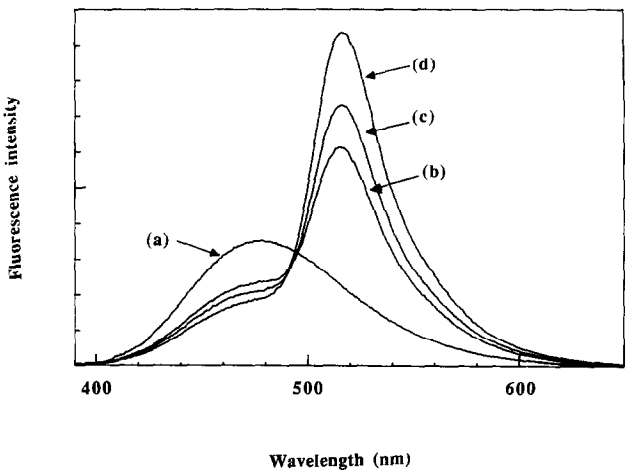

Fig. 3. Fluorescence emission spectra of labeled I-form α_2M. (a) I-form α_2M with dansyl label attached to one cysteine and the other cysteine blocked with iodoacetamide; (b) I-form α_2M with 1.0 dansyl labels and 0.51 fluorescein labels; (c) I-form α_2M with 1.0 dansyl label and 0.63 fluorescein labels; (d) I-form α_2M with 1.0 dansyl label and 0.83 fluorescein labels. Spectra were recorded at equal concentrations of α_2M (1.0 μM) and identical spectrometer settings. All species have identical amounts and distributions of dansyl label, since they all derived from the same labeling reaction with 1,5-I-AEDANS.

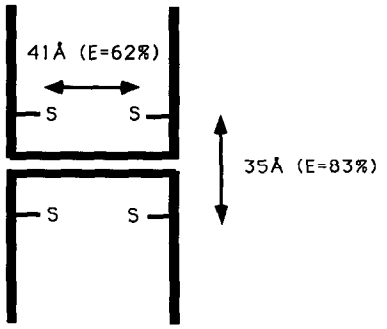

Fig. 4. Schematic representation of the location of the four Cys-949 residues in an α_2M tetramer based upon measurements made here and previously [1]. The distances given are for separations between the fluorophores attached to the cysteine residues, and are based on $R(2/3)$ values. Efficiencies of fluorescence energy transfer are given in parentheses.

levels and distribution of dansyl was calculated as 33%, 40% and 46.4%, respectively, after subtraction of the small contribution from fluorescein emission in the region of the dansyl spectrum. Using the non-random distribution of dansyl and fluorescein labels expected from the differential reactivity of the two Cys-949 residues reported above for I-form α_2M, we calculated efficiencies of fluorescence energy transfer of 64.7%, 63.5%, and 55.9% for the samples containing 0.51, 0.63, and 0.83 fluorescein labels, respectively. These correspond to separations of 39.3 Å, 39.7 Å, and 41.8 Å, respectively, with an average of 40.3 Å, using the calculated isotropic $R_0(2/3)$ value of 43.5 Å. Using maximum and minimum values of κ^2, calculated from anisotropy measurements, maximum and minimum values of R_0 of 55.7 Å and 33.5 Å were determined (identical to values found previously for dansyl/fluorescein-labeled α_2M species [1]), which resulted in a range of acceptable values for the separation R of 41.3 ± 10 Å.

4. DISCUSSION

By incorporating both donor and acceptor fluorophores into the same half of α_2M, defined operationally as the half that reacts with Sepharose-linked chymotrypsin to give I-form α_2M, we have determined the separation between these two Cys-949-bonded fluorophores as 41.3 ± 10 Å. This value, taken together with our earlier measurement of 35 ± 9 Å for the separation between the same fluorophores attached to Cys-949 residues in *different* halves of α_2M, shows that the four Cys-949 residues in α_2M are centrally placed in the α_2M tetramer, at the corners of a rectangle of almost equal sides (Fig. 4). The separation between the cysteines, rather than between the attached fluorophores, may need to be increased by about 10–15 Å depending on the relative orientation of the fluorophores. The relatively large lateral separation reported here represents the minimum internal diameter of the hollow proteinase trap and explains how α_2M can accommodate quite

large proteinases such as plasmin [15] with a diameter for the light chain of about 50 Å [16], and even allow rotation of complexed but not cross-linked chymotrypsin within the trap [17].

The uncertainty in κ^2 results in a relatively large uncertainty in the separation between the fluorophores. However, the *ratio* of the separations of Cys-949 residues within a half molecule to those in different half molecules is likely to be close to the value of 41:35, since, whatever the value of κ^2 is, it is likely to be the same or very similar for all of the species examined here and in our earlier studies [1,14]. There is also uncertainty in the derived separation from not knowing precisely the distribution of dansyl and fluorescein fluorophores between the two cysteine residues.

The surprising finding of differential reactivity of the two Cys-949 residues, although presently not explicable, greatly facilitated the present study, since it allowed preparation of doubly-labeled α_2M in which each α_2M molecule contained one dansyl and one fluorescein. Further support for such a non-random distribution is provided by comparison of the observed quenching of dansyl fluorescence by fluorescein with the maximum possible values if the first-reacted dansyl label had distributed randomly amongst the two potential sites of attachment. For random distribution, reductions of only 25.5%, 31.5%, and 41.5% would have been observed for the samples containing 0.51, 0.63, and 0.83 fluoresceins, respectively, even if the efficiency of energy transfer had been 100%. These compare with observed values of 33, 40, and 46.4%. Since it can easily be shown that the maximum percentage reduction (for $E = 100\%$) in dansyl fluorescence for random label distribution does not depend on the degree of dansyl incorporation, but only on the stoichiometry of the acceptor fluorophore, the discrepancy between observed and theoretical values strongly imply that random distribution did not occur. Although it is thus clear that dansyl labelling was not random, and that there is a large difference in reactivity of the first and second SH groups, there is the possibility that some α_2M molecules contained two dansyl labels and some contained none. However, the approximately constant extrapolated value for the efficiency of quenching at different fluorescein stoichiometries suggests that this is not a major source of error.

Whether the observed differential reactivity is a property of unreacted I-form α_2M, or is induced by chemical modification of one of the two Cys-949 residues is not at present known. A detailed investigation of this is warranted, since it may shed further light on the role that thiol ester cleavage plays in the conformational changes that α_2M undergoes upon reaction with proteinase.

Acknowledgements. This work was supported by NIH grants HD28187 and ES00267. We thank Drs. Steven Olson and Phil Patston for helpful discussions and critical reading of the manuscript, and Dr. Paul Bock for use of his SLM 8000 fluorimeter.

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