Structural analysis of acute-phase α_2 -macroglobulin

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High resolution images of rat acute-phase α_2 -macroglobulin (AP α_2 M) have been obtained by using dark-field electron microscopy. No staining or artifact-inducing procedures were used. Analysis of unfiltered electron microscope plates, exposed to minimal electron beam radiation, revealed highly contrasted particles of variable morphology with dimensions of approx. 19 nm $\times$ 14 nm. An electron-dense core with four to six projections could be seen. Two-fold symmetry was evident in selected images, supporting the four-subunit composition of the protein. Image processing and filtering confirmed the presence and configuration of the projections by demonstrating exact molecular dimensions of 16 nm $\times$ 9.5 nm and a shape with six projections like that of the Russian letter Ж. SDS/polyacrylamide-gel electrophoresis revealed that this molecule was in the proteinase-bound form. C.d. data revealed a surprisingly low content of α -helical secondary structure (12%) and an atypically large content of β -form structure (33%). Comparison of the amino acid compositions of AP α_2 M and human α_2 -macroglobulin indicated a high degree of homology between the two molecules. It is concluded that the conformation of rat AP α_2 M, both at the molecular and secondary structural levels, is strikingly similar to that of human α_2 -macroglobulin.

INTRODUCTION

Human α_2 -macroglobulin (h α_2 M) and rat acute-phase α_2 -macroglobulin (AP α_2 M) are high- M_r glycoproteins that have been demonstrated by functional (Westrom, 1979; Gonias *et al.*, 1983) and immunological (Heim, 1968) criteria to belong to a family of macroglobulins that have been implicated as modulators in many immunological and inflammatory processes. For example, AP α_2 M is markedly elevated in serum during pregnancy (Panrucker *et al.*, 1983) and following an experimentally induced acute-phase inflammatory reaction (Panrucker & Lorscheider, 1982a; Heim & Lane, 1964). h α_2 M is a major serum component (James, 1980) which consists of four polypeptide chains, each of 185 kDa, which interact with one another by interchain disulphide bonds and noncovalent forces (Starkey, 1979). This macroglobulin strongly binds to trypsin and other endopeptidases (Starkey, 1979) as well as to a wide range of metallic ions and immunological modulators (James, 1980). Electron microscopy studies of stained h α_2 M (Schram & Schram, 1982; Tapon-Brethaudiere *et al.*, 1985) show distinctive molecular conformations with and without bound trypsin. On the basis of the functional similarities between AP α_2 M and h α_2 M, it was of interest to establish the structural relationship between these two macromolecules.

The primary objective of the present investigation was to apply dark-field electron microscopy (DFEM) and image processing, as we have done for a smaller protein (Luft & Lorscheider, 1983), to elucidate the structure of AP α_2 M. Because of the similarities between AP α_2 M and h α_2 M, another objective was to investigate and compare secondary conformational features of these two macromolecules by using c.d. and amino acid composition data.

MATERIALS AND METHODS

Specimen preparation

AP α_2 M was isolated and highly purified from fresh serum of croton oil-induced rats by a two-step procedure involving gel filtration and negative immunoaffinity chromatography (Panrucker & Lorscheider, 1982b). Purity was assessed by native discontinuous polyacrylamide-gel electrophoresis (Ornstein, 1964; Davis, 1964) and SDS/polyacrylamide-gel electrophoresis (O'Farrell, 1975). To determine if our AP α_2 M was bound to serine proteinases, we examined the fragments obtained under reducing SDS/polyacrylamide-gel electrophoresis. M_r values were calculated by comparison with reference standard proteins (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and applying linear regression analysis. AP α_2 M was incubated at room temperature with a 2:1 molar ratio of purified trypsin for 10 min. Approx. 50 μ g of protein was loaded per lane and gels were stained with Coomassie Blue. Protein was diluted to a concentration of 20 μ g/ml in distilled water and, for another preparation, into a 0.2 M-sodium borate buffer (pH 8.9). Each preparation was applied to an electron microscope grid allowing molecules to adsorb onto an ultra-thin carbon film prepared by indirect evaporation of carbon onto freshly cleaved mica (Johansen, 1974). After 4–5 min, the grids were washed with 20 drops of distilled water to remove excess AP α_2 M and air dried at room temperature. No staining procedure was used in the preparation of the specimens.

Dark-field electron microscopy

DFEM (Dupouy, 1967) was performed using a Philips transmission EM-300 in beam-tilt mode at magnifications of $\times 31\,920$ and $\times 51\,300$. Electron micrography with astigmatism correction was employed as we have

Abbreviations used: AP α_2 M, rat acute-phase α_2 -macroglobulin; h α_2 M, human α_2 -macroglobulin; DFEM, dark-field electron microscopy.

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previously described (Luft & Lorscheider, 1983), with emphasis on minimal electron beam radiation exposure.

Selection of molecules

Electron microscope plates were scanned by stereomicroscope ($\times 15$) for a contrasting and repeating structure corresponding to a size-range of 5–2300 nm based on globular or linearly arranged $AP\alpha_2M$ molecules calculated to contain 5988 amino acid residues (Starkey, 1979; Luft & Lorscheider, 1983). $AP\alpha_2M$ molecules were easily identified over the background noise contributed from the carbon support film. The locations of individual $AP\alpha_2M$ molecules were marked and photographic enlargements made as previously described (Luft & Lorscheider, 1983).

Image processing

Individually enlarged molecules were placed into a 3×3 array (nine individual images) and this same array was repeated nine times, forming a composite array of 81 molecules allowing enhanced uniform averaging (Ottensmeyer *et al.*, 1977). In another composite array, every second 3×3 array was alternately rotated 180° in the same plane and this composite array was subsequently processed. The optical image processing technique which we employed on these composite arrays is called 'Filtering of Arrays of Images in Reciprocal Space (FAIRS)' which filters out random background noise while permitting true averaged signal reconstruction of the molecule. Our laboratory has described this process in detail (Luft & Lorscheider, 1983). Briefly, a 6 mW spatially filtered He-Ne laser beam was passed through a photonegative of the composite array and the diffraction pattern that resulted was optically filtered by a fenestrated metal filter having a reciprocal lattice arrangement of holes which matched the composite array. The filtered diffraction pattern was reconstructed by a lens to produce a new array of averaged and noise-reduced molecular images. The filtered array was focused onto a 35 mm camera back and photographed with Kodak Technical Pan film 2415.

C.d. and amino acid composition analysis

C.d. spectra of $AP\alpha_2M$ were recorded on a Jasco (Easton, MD, U.S.A.) J500C Spectropolarimeter. Protein samples were dissolved in 0.2 M-sodium borate buffer, at pH 8.9 to prevent aggregation, to a concentration of 122 $\mu\text{g/ml}$ as determined by the Waddell (1956) method. Spectra were recorded over a 205–250 nm range, with each 1 nm interval ellipticity being converted into mean residue ellipticity (Reed *et al.*, 1975). The plotted data represent an average of four runs after baseline subtraction using the least squares method of Chen *et al.* (1974). The proportions of α -helix, β -form and random coil were determined by fitting the data to a standard curve using the basis spectra of Chen (Fasman, 1976) and applying a curve-fitting program written in BASIC on a Honeywell DPS 8107M computer. A molecular mass of 716 kDa (Gordon, 1976) with an 8% carbohydrate correction (Björk & Fish, 1982) was used in the calculations. Purified rat $AP\alpha_2M$ was hydrolysed in 6 M-HCl at 110°C for 20 h and the hydrolysate was analysed on a Beckman 121 M amino acid analyser equipped with a Beckman system AA integrated data reduction system (Beckman Instruments, Palo Alto, CA, U.S.A.). No corrections were made for hydrolytic losses

of serine or threonine. Tryptophan was not determined. Discrimination between glutamic acid/glutamine and aspartic acid/asparagine content could not be made. The relatedness of our $AP\alpha_2M$ to $h\alpha_2M$, isolated by Sottrup-Jensen *et al.* (1979), was estimated by calculating a ΔQ value from the respective amino acid compositions, both of which were uncorrected for hydrolytic losses. The analytical procedure described by Marchalonis & Weltman (1971) was used to calculate a relative homology index between proteins based on composition data.

RESULTS

Fig. 1 shows photographs of exposed electron micrograph plates. The bar represents 195 nm. Specimen preparation using $AP\alpha_2M$ diluted with distilled water produced extensive aggregation of molecules (Fig. 1a) with no discernible structure. Preparations diluted with 0.2 M-borate buffer (pH 8.9) gave preparations having isolated molecules of a consistent size and little aggregation (Fig. 1b). Only electron image plates appearing to show optimal focus, no astigmatism and no specimen stage drift were used for further analysis. The specimen regions used were not pre-irradiated prior to recording on film. Fig. 2 presents enlargements of selected areas from three different plates, each showing a molecule with symmetry and in a size range of 15–20 nm. The bar represents 5 nm.

Fig. 3(a) shows the original composite array of unfiltered molecules while Fig. 3(b) shows the same array after image processing. An enlargement from Fig. 3(b) is shown in Fig. 4(a), while Fig. 4(b) is an enlarged molecule from the second, rotated, composite array. Some structural features of $AP\alpha_2M$ are vaguely defined in the unfiltered molecules, notably projections emanating from an electron-dense core (Fig. 2). These projections became more distinct when the signal-to-noise ratio was increased by optical filtering (Figs. 4a and 4b). Fig. 4(a) shows distinct symmetrical images not unlike the Russian letter Ж. Arms are notably dense in the upper left quadrant and lower right quadrant and less so in the remaining two quadrants. Centrally, two electron-dense arms protrude, although extending outwards to a lesser degree. Recognizing the two-fold symmetry (rotation axis perpendicular to the plane of the print) in Fig. 4(a), rotation within the composite array clarifies the above features to an even greater extent (Fig. 4b). Both the original and rotated images measure $16\text{ nm} \times 9.5\text{ nm}$ in their rectangular dimensions.

Fig. 5 presents the c.d. spectrum for $AP\alpha_2M$, with the total α -helix, β -form, and random coil content (inset). The secondary conformation of $AP\alpha_2M$ at pH 8.9 shows α -helix content of 12%, β -form content of 33%, and random coil content of 55% with inter-run S.E.M. variabilities of 1.1%, 0.8% and 1.8% respectively. Amino acid composition data for $AP\alpha_2M$ and $h\alpha_2M$ are contained in Table 1. The computed ΔQ value, comparing amino acid composition analyses of $AP\alpha_2M$ and $h\alpha_2M$, was 18.5, a low value indicating a high degree of homology.

Fig. 6 shows SDS/polyacrylamide-gel electrophoresis separations of purified trypsin, $AP\alpha_2M$ -trypsin (1:2), $AP\alpha_2M$ alone and M_r standard markers. Lane 1 shows the main trypsin band (24 kDa) with autolytic breakdown products below it. Lane 2 shows a main 167 kDa subunit

Fig. 1. Electron micrograph of $AP\alpha_2M$ molecules in distilled water (a) and in a 0.2 M-borate buffer at pH 8.9 (b)
The bar represents 195 nm.

Fig. 2. Three enlarged $AP\alpha_2M$ molecules from plates such as that shown in Fig. 1(b)
The bar represents 5 nm.

of $AP\alpha_2M$, along with proteolytic fragments and unbound trypsin. Lane 3 shows the main 167 kDa $AP\alpha_2M$ subunit and a second 84 kDa subunit, characteristic of $AP\alpha_2M$ that has bound serine proteinases (Harpel, 1973). M_r markers are seen in lane 4, ranging between 14.4 and 200 kDa. A single band (not shown) of purified $AP\alpha_2M$ appears on native discontinuous polyacrylamide-gel electrophoresis (Panrucker & Lorscheider, 1982b).

DISCUSSION

The molecular conformation of $AP\alpha_2M$ has been rapidly elucidated by DFEM, image processing, and c.d. to approximately 2 nm resolution.

As can be seen from Fig. 1(a), preparations of $AP\alpha_2M$ with distilled water produced clumps of aggregated molecules, whereas the use of 0.2 M-borate buffer (pH 8.9) gave suitable preparations characterized by well-dispersed and highly contrasted molecules (Fig. 1b). It seems probable that at the pH and ionic strength of the borate buffer, individual $AP\alpha_2M$ molecules carry a net negative charge that prevents aggregation.

Close examination of individual unfiltered molecules (Fig. 2) consistently revealed an electron-dense core with four to six 'arms' protruding from the core. Such an observation is very similar to the stained $h\alpha_2M$ bright-field electron microscope images of Schram & Schram (1982) who showed similar size particles that revealed, upon computer image stacking, six domains

Fig. 3. Original composite array of unfiltered AP α_2 M molecules (a) and the corresponding filtered array using the FAIRS system (b). The bar represents 50 nm.

Fig. 4. Single, filtered and enlarged AP α_2 M molecules from the original composite array (a) and from a second rotated array of molecules (b).

The bar represents 4 nm.

protruding symmetrically from the centre of the molecule. The two-fold symmetry observed for AP α_2 M in the present study is consistent with the four-subunit model proposed by Harpel (1973) for h α_2 M.

Tapon-Brethaudiere *et al.* (1985) and Schram & Schram (1982) have observed a difference between h α_2 M with and without bound trypsin. Our filtered AP α_2 M images are most similar to the trypsin-bound form of h α_2 M. Both SDS and native gels confirm that our protein is homogeneous. We conclude that our AP α_2 M has serine proteinases bound to it, because 167 kDa and 84 kDa

bands are evident under reducing conditions (Fig. 6, lane 3). Furthermore, the appearance of proteolytic fragments when AP α_2 M is incubated with trypsin supports our interpretation that AP α_2 M is already proteinase-bound, and thus unable to function in an anti-proteolytic capacity (Fig. 6, lane 2). Preliminary fluorescence emission spectroscopy (Bjork & Fish, 1982) performed on our AP α_2 M preparation supports its proteinase-bound conformation (G. J. Beitel, A. J. Luft, D. E. Panrucker & F. L. Lorscheider, unpublished work).

Individual molecules of AP α_2 M imaged by DFEM

Table 1. Comparison of amino acid composition of rat AP α_2 M with human α_2 M

Amino acid	Composition (mol/100 mol) in:	
	AP α_2 M	h α_2 M
Asx	8.56	7.93
Thr	6.84	6.28
Ser	7.79	7.31
Glx	12.41	12.28
Pro	4.91	5.72
Cys	0.95	1.93
Gly	7.92	6.34
Ala	8.90	6.28
Val	8.12	10.00
Met	1.89	1.66
Ile	3.56	3.72
Leu	9.64	9.03
Tyr	3.26	3.72
Phe	5.48	4.14
His	2.03	2.62
Lys	6.09	6.21
Arg	3.66	3.17
Trp	N.D.*	N.D.*

* Not determined.

Fig. 5. C.d. spectrum of AP α_2 M with the α -helix, β -form, and random coil content (inset)

show some structural variation due to noise contributions. Image processing of composite arrays reduced this variation through averaging and increasing the signal-to-noise ratio by a factor of 3 as described by Ottensmeyer *et al.* (1977). Rotation with image processing, while increasing the averaging of the original images, did not improve the signal-to-noise ratio, but did increase the delineation of the two central 'arms'. The fact that the conformation of the molecule remained essentially unchanged through the rotation shows that AP α_2 M does possess a two-fold rotation axis. It is interesting to note that while symmetry is apparent, it is not as uniform as

Fig. 6. SDS/polyacrylamide-gel electrophoretic separations

Lane 1, purified trypsin; lane 2, AP α_2 M-trypsin (1:2 molar ratio); lane 3, purified AP α_2 M; lane 4, M_r standard markers (myosin, 200 kDa; β -galactosidase, 116.25 kDa; phosphorylase *b*, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa).

one might expect. This may represent skewed averaging of certain arms either due to the molecule's inherent flexibility, or to mobility in the molecule while being exposed to the electron beam. The blurring of the filtered image may also reflect error in alignment, some radiation damage or specimen stage drift. In any case, these factors may partially limit resolution in the DFEM system when applied to macromolecules. We believe that the resolution obtained for AP α_2 M is at least 2 nm, although theoretically DFEM/FAIRS will allow a 0.5 nm resolution on smaller molecules (Ottensmeyer *et al.*, 1978). Although we could not ascertain the third dimension (thickness) of AP α_2 M we believe it to be less than the width or length because of its preferred orientation on the thin carbon film.

C.d. at pH 8.9 revealed that AP α_2 M had an unusually low amount of α -helix and an atypically (Rawn, 1983) high amount of β -form structure. Since c.d. studies were conducted under non-physiological conditions, it is possible that denaturation via disruption of α -helices could have occurred. However, our study has shown that AP α_2 M has an almost identical secondary structure to that reported for h α_2 M at pH 8.0 (Richman & Verpoorte, 1981), and Sottrup-Jensen *et al.* (1979) have shown an amino acid composition for h α_2 M similar to that for AP α_2 M. The filtered DFEM images of AP α_2 M and h α_2 M are remarkably similar, and this similarity extends to the amino acid compositions as shown by the low SAQ value of 18.5 obtained from the interspecies comparison of amino acid composition data. By way of comparison, the SAQ value for highly conserved molecules such as horse and human cytochrome *c* is 26 (Marchalonis & Weltman, 1971). Finally, we speculate that the relatively high β -form content, as determined by c.d., may be part of the non-covalent forces required for AP α_2 M subunit aggregation. β structures would allow subunit-subunit interaction of a non-covalent type by

forming parallel/anti-parallel β -pleated sheets (Walton & Blackwell, 1973).

In this study, we have shown that the proteinase-bound form of rat AP α_2 M is a compact molecule shaped like the Russian letter Ж which shows striking structural homology by several criteria to the corresponding form of h α_2 M. The highly conserved structure of these α_2 -macroglobulins supports their fundamental role in common physiological processes through mammalian species.

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