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Characterization of a half-molecular fragment obtained by reduction of human α_2-macroglobulin with dithiothreitol

A small-angle X-ray and neutron scattering investigation

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Abstract. A half-molecular fragment of α_2-macroglobulin has been prepared by reducing and alkylating the inter-subunit disulfide bonds in the tetrameric α_2-macroglobulin molecule with 1 m*M* dithiothreitol (40 min) and 3 m*M* iodoacetamide (40 min). Further purification was made by gel chromatography and the homogeneous population of half-molecules has been characterized by the techniques of small-angle X-ray and neutron scattering. The radii of gyration found by the two methods are 57.0 and 58.0 Å, respectively. The match point, obtained by neutron scattering from solutions with different H_2O/D_2O ratios, is at 43% D_2O; the data are consistent with a particle having a higher scattering density at large distances from the particle centre. From the X-ray and neutron intensities scattered at zero angle, the specific volume was determined to be 0.73 cm^3/g at + 5 °C and the molecular weight to be 390,000; the latter value is associated with a relatively large error due to the uncertainty in the concentration determination. Shape analysis indicates that the best-fitting scattering-equivalent three-axial bodies are oblate shaped, with two of their axial dimensions about three to four times larger than the third one. From the volume of the best-fitting scattering-equivalent three-axial bodies, 0.72×10^6 Å^3, we obtain a water content equal to 0.38 g H_2O/g protein (dry weight).

Key words: α_2-macroglobulin, subunit structure, small-angle X-ray scattering, small-angle neutron scattering, supercooling

Introduction

Human α_2-macroglobulin is a plasma glycoprotein of molecular weight about 720,000 containing about 10% carbohydrate and with a physiological plasma concentration of approximately 2 mg/cm^3. $\alpha_2 M$ has the unique property of acting as a molecular trap for a wide range of proteolytic enzymes. The interaction between $\alpha_2 M$ and proteases involves a conformational change of $\alpha_2 M$ which results in an entrapment of the protease molecule. The conformational change leads to a rapid clearance of the $\alpha_2 M$-protease complex via highly discriminative receptors (for recent reviews, see Barrett 1981; Van Leuven 1982; Travis and Salvesen 1983).

An analysis of the subunit composition of $\alpha_2 M$ indicated that the molecule is composed of four identical subunits (Harpel 1983; Barrett et al. 1979). In spite of its important role as a protease inhibitor very little is known about the inhibition reaction on a molecular level. Furthermore, little is known about the three-dimensional shape of $\alpha_2 M$ and about the organisation of the subunits in the molecule. In order to provide more information regarding these questions we have started small-angle neutron and X-ray scattering investigations of fragments obtained by dissociating $\alpha_2 M$. This report deals with an investigation of the half-molecule of $\alpha_2 M$ obtained by reducing and alkylating the inter-subunit disulfide bonds of the $\alpha_2 M$ tetramer followed by purification using gel chromatography.

Experimental

Preparation of $\alpha_2 M$

$\alpha_2 M$ was isolated from fresh human plasma using a slight modification of the method described by Harpel (1976); the modifications will be described

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Abbreviations: SANS = small-angle neutron scattering, SAXS = small-angle X-ray scattering, $\alpha_2 M = \alpha_2$-macroglobulin, DTT = dithiothreitol, Tris = tris(hydroxymethyl)aminomethane in more detail elsewhere (Sjöberg et al., to be published). No impurities could be detected when a sample of $\alpha_2 M$ was subjected to agarose gel electrophoresis and crossed immunoelectrophoresis (Ganrot 1972).

Preparation of $\alpha_2 M$ half-molecules

$\alpha_2 M$ dissolved in a 0.05 M Tris-HCl buffer, pH 8.1, was reacted with 1 mM DTT (40 min) and then with 3 mM iodoacetamide (40 min) at 22 °C under non-denaturing conditions (Barrett et al. 1979). This procedure did not produce a homogeneous population of half molecules so a purification step had to be made using Sephacryl S 300 (Pharmacia) gel chromatography in 0.05 M Tris-HCl buffer, pH 8.1, containing 1 mM DTT and 3 mM iodoacetamide. Protein was detected by UV absorption at 280 nm. Fractions were analysed by polyacrylamide gel electrophoresis (Hall and Roberts 1978), pooled and concentrated by exposing a dialysis bag containing the solution to low pressure. The concentration of the half-molecule was determined by measuring the absorbance at 280 nm using the value of $A^{1\%}_{1\,\mathrm{cm}} = 9.1$. All the SAXS and SANS measurements were made in a 0.05 M Tris-HCl buffer, pH 7.8 containing 1 mM DTT and 3 mM iodoacetamide. The samples were checked both before and after the small-angle measurements using polyacrylamide gel electrophoresis.

In order to check that the preparation procedure caused no serious changes of the samples, tests have been made where we reassociate the half molecules to a total $\alpha_2 M$ molecule and test for its activity. At least in those cases in which we exclude the iodoacetamide step, it is possible to reassociate the half-molecules to a tetramer which is active with respect to trypsin binding (Ganrot 1966) and which gives a SAXS curve identical to that obtained for $\alpha_2 M$ not subject to dissociation.

Small-angle X-ray scattering measurements

The SAXS data were recorded with the camera developed by Kratky and Skala (1958) as described by Sjöberg and Pap (1985). The concentration range was from 3 to 10 mg/cm^3.

In order to increase the stability of the samples in the X-ray beam, all measurements, except those which were used for determining the specific volume (see below), were made using supercooled water solutions at − 10 °C (Sjöberg and Pap 1985). Apart from the marked increase in stability of the samples and the slight increase in signal-to-background ratio, no difference could be detected in the basic shape of the scattering curve at − 10 °C compared with that at room temperature.

Small-angle neutron scattering measurements

Data were collected using the SANS-instrument at the Risø National Laboratory, Denmark (Kjems et al. 1985). The sample-to-detector distance was 300 cm and the two neutron wavelengths 0.40 and 1.5 nm ($\Delta\lambda/\lambda = 18\%$ at FWHM) were used. Scattering intensities, normalised to the primary beam monitor, were measured by a two-dimensional position-sensitive detector that encodes the position of the detected neutrons and sums them into elements of a 64 × 64 array. The data were corrected for background by using the intensity profiles obtained from the quartz cell filled with dialysis buffer, an empty cell and with the primary beam blocked with boron plastic. Next, the data were corrected for non-linearity in detector response and referenced on an absolute scale by dividing through with the incoherent scattering from a one mm water sample (Jacrot and Zaccai 1981). All data were collected at 5 °C using six different D_2O concentrations and protein concentrations in the range from 1.5 to 13 mg/cm^3. D_2O concentrations were determined by measuring both the transmission of neutrons through the sample and the density (Inoue et al. 1981) using a digital density meter (Kratky et al. 1969).

Data analysis and results

The analysis of the SAXS data followed conventional procedures involving desmearing (Glatter 1974) and Guinier plots to determine the radius of gyration and forward scattering.

From the neutron data the radius of gyration and forward scattering were obtained by fitting a Gaussian function to the radial intensity function at small values of momentum transfer. The effect of the relatively broad wavelength distribution ($\Delta\lambda/\lambda = 18\%$ at FWHM) was considered by smearing the Gaussian function with the appropriate wavelength distribution function. A check of the procedure mentioned above was made by a more direct approach where individual intensity values from the 64 × 64 detector array were fitted directly to the Gaussian, without going *via* the radial intensity function. In this latter case the geometrical resolution effect due to the dimensions of the primary beam was also included within the calculations. This effect was, however, found to be negligible and the two methods agreed within the standard deviations.

A small concentration-dependent inter-particle scattering effect was eliminated by extrapolating all parameters to zero concentration.

The radius of gyration

The values of the radii of gyration, R, obtained by neutron scattering, were analysed according to (Ibel and Stuhrmann 1975)

$$R^2 = R_c^2 + \alpha/\Delta\varrho - \beta/\Delta\varrho^2 . \qquad (1)$$

Here $\Delta\varrho$ is the difference in scattering density between the macromolecule and the solvent and R_c is the radius of gyration at infinite contrast. In a plot of R^2 as a function of $1/\Delta\varrho$, a slightly curved result is obtained (Fig. 1). By fitting the constants of Eq. (1) to the experimental data with the help of a non-linear least-squares procedure we obtained $R_c = 58.0 \pm 0.3$ Å, $\alpha = (94 \pm 27)\ 10^{-6}$ and $\beta = (79 \pm 38)\ 10^{-12}$ Å^{-2}. The positive value of α means that there is less than average scattering density close to centre of the particle. A positive β, different from zero, means that the centres of gravity of the scattering density and volume do not coincide (Ibel and Stuhrmann 1975).

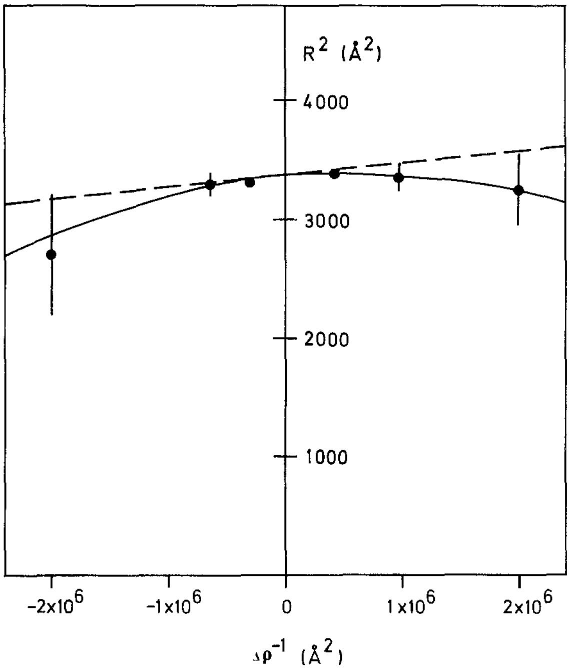

Fig. 1. The square of the radius of gyration, R^2, obtained by neutron scattering as a function of the inverse of the contrast, $\Delta\varrho$. The solid line corresponds to Eq. (1) with $R_c = 58.0$ Å, $\alpha = 94 \times 10^{-6}$ and $\beta = 79 \times 10^{-12}$ Å^{-2}. The broken line is the corresponding function with $\beta = 0$

The radius of gyration at infinite contrast, R_c, is equal to 58.0 Å. The radius of gyration obtained from the SAXS measurements was found to be 57.0 Å.

Zero angle scattering of neutrons and X-rays from H_2O solutions and the relation to molecular weight and specific volume

The general equation for the normalised zero angle scattering of both neutrons and X-rays is

$$I(0) = M_r N_A\, c\, d(\Sigma\, b/M_r - \varrho_s V/M_r)^2 , \qquad (2)$$

where M_r is the molecular weight, N_A Avogadro's constant, c the concentration, d the sample thickness, b the coherent scattering length, ϱ_s the scattering density of the solvent and V the dry volume of the particle. The normalised intensities scattered at zero angle, $I_{\mathrm{SANS}}(0)$ and $I_{\mathrm{SAXS}}(0)$, were obtained as described above. By using the appropriate scattering lengths for neutrons and X-rays together with the compositions of the protein and carbohydrate parts as given by Sottrup-Jensen et al. (1984) and Dunn and Spiro (1967), respectively, we obtain for water solutions

$$I_{\mathrm{SANS}}(0) = 10^{-28} N_A M_r\, c\, d(2.255 + 0.9096\, v_2)^2 \qquad (3)$$

and

$$I_{\mathrm{SAXS}}(0) = 10^{-28} N_A M_r\, c\, d(15.00 - 15.55\, v_2)^2 , \qquad (4)$$

where v_2 is the specific volume of the solute.

Equations (3) and (4) were solved for the two unknowns M_r and v_2. In fact, in order to eliminate the error in the concentration determination from the value of the specific volume, $I_{\mathrm{SANS}}(0)$ and $I_{\mathrm{SAXS}}(0)$ were determined using samples with the *same* concentration taken from the *same* stock solution. Thus $M_r c$ can be eliminated from Eqs. (3) and (4) and v_2 can be determined without even knowing the concentration. However, in order to obtain M_r the concentration must be known. The result, obtained using data recorded at 5 °C, was $v_2 = 0.73$ cm^3/g and $M_r = 390{,}000$.

The average specific volume calculated from the composition of the protein and carbohydrate parts (Sottrup-Jensen et al. 1984; Dunn and Spiro 1967), using the data given by Cohn and Edsall (1943) and Perkins and Miller (1981), is equal to 0.73 cm^3/g.

Zero angle scattering of neutrons as a function of D_2O content and the number of exchangeable protons

Figure 2 shows a plot of the square root of the intensity at zero angle, corrected for concentration, transmission and sample thickness, as a function of

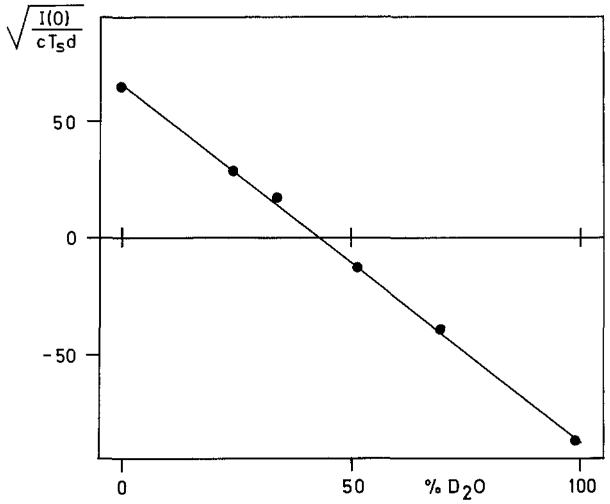

Fig. 2. Square root of the neutron intensity at zero angle, corrected for concentration, transmission and sample thickness, as a function of the percentage of D_2O in the solvent. The match point is at 43% D_2O

Table 1. Typical results of the least-squares fit of three-axial bodies to the SAXS data; a, b and c are the semiaxes and L is the total length. The volume, V_{calc}, and radius of gyration, R_{calc}, are the values calculated from the geometrical dimensions of the bodies. U is the average difference between the experimental and calculated intensity values (Sjöberg 1978)

Model	Dimensions [Å]	V_{calc} [Å^3]	R_{calc} [Å]	U [%]
Prolate ellipsoid of revolution	$a = b = 37.0$ $c = 167$	960,000	78.3	6.03
Oblate ellipsoid of revolution	$a = 22.5$ $b = c = 85.5$	690,000	55.0	1.94
Ellipsoid	$a = 22.9$ $b = 71.6$ $c = 104$	710,000	57.4	1.84
Elliptic cylinder	$a = 22.0$ $b = 69.3$ $L = 150$	720,000	56.5	1.85
Elliptic cylinder	$a = 22.1$ $b = 96.4$ $L = 107$	720,000	58.3	1.80

the percentage of D_2O in the solvent. As expected, we obtain a straight line; the match point is at 43% D_2O. From the composition of $\alpha_2 M$ (Sottrup-Jensen et al. 1984; Dunn and Spiro 1967) we obtain

$$\Sigma\, b/M_r = (2.255 + 1.605\, PX)\, 10^{-14}, \qquad (5)$$

where b is the scattering length and $100\,X$ is the percentage of D_2O in the solvent; it is assumed that $100\,P$% of the hydrogen atoms bound to oxygen and nitrogen exchange in proportion to X. At the match point

$$\Sigma\, b/M_r = \varrho_s\, V/M_r, \qquad (6)$$

where V is the dry volume of the molecule and $\varrho_s = (-0.548 + 6.97\,X)\,10^{10}\,\mathrm{cm}^{-2}$ is the scattering length per unit volume of the solvent.

By using the value of the partial specific volume, $v_2 = 0.73\ \mathrm{cm}^3/\mathrm{g}$, determined in the previous section, together with Eqs. (5) and (6) we obtained $P = 1.02$. That is, our data can be explained if we assume that approximately all of the hydrogen atoms bound to oxygen and nitrogen are exchangeable. It should be noted, however, that the determination of P is rather inaccurate. A small error in X will produce a large error in P, for instance, $X = 0.42$ corresponds to $P = 0.92$.

Models for the particle shape

A spherical particle with a molecular weight equal to 390,000 and a water content of, say, 0.3 g H_2O/g protein (dry weight), would correspond to a radius of gyration of about 42 Å. Thus, we can immediately conclude that the half-molecule of $\alpha_2 M$ must have a more elongated shape than a sphere. A loose structure such as a gaussian coil (e.g. Kirste and Oberthur 1982) is unable to explain the data. A more detailed shape analysis has been performed using a nonlinear least-squares procedure (Sjöberg 1978). Typical results of the fitting procedure are given in Table 1. It follows that the best-fitting scattering-equivalent three-axial bodies are oblate shaped with two dimensions about three to four times larger than the third one.

From the volumes, V_{calc}, obtained for the best-fitting models in Table 1, about 0.72×10^6 Å^3, together with the molecular weight and specific volume we obtain a water content equal to 0.38 g H_2O/g protein (dry weight).

Discussion

In this work the half-molecule of $\alpha_2 M$ has been prepared in pure form and characterized by the SAXS and SANS methods. The two methods complement each other and it is an advantage to record both types of data. For instance, by using the $I(0)$ values obtained by the two methods the specific volume of the solute can be obtained even if the concentration is unknown, or known only on a relative scale. A summary of the molecular parameters obtained are given in Table 2.

The half-molecule of $\alpha_2 M$ has recently been prepared (Gonias and Pizzo 1983) by treating $\alpha_2 M$ with 0.5 mM DTT (1 h) and 1.3 mM iodoacetamide (40 min). In this work similar conditions, 1 mM

Table 2. Molecular parameters for the half-molecule of $\alpha_2 M$ deduced from small-angle X-ray and neutron scattering data

Radius of gyration	57.0 Å (SAXS), 58.0 Å (SANS)
Molecular weight	390,000
α	94×10^{-6}
β	79×10^{-12} Å^{-2}
Match point	43% D_2O
Specific volume [+ 5 °C]	0.73 cm^3/g
Volume	0.72×10^6 Å^3
Water content [+ 5 °C]	0.38 g H_2O/g protein (dry weight)

DTT (40 min) and 3 mM iodoacetamide (40 min), were used. Since the SAXS and SANS methods are rather sensitive to impurities, such as, for instance, undissociated tetramers, the reduction and alkylation step was followed by purification using gel chromatography. The chemical properties of the half-molecule have been extensively characterized in the work of Gonias and Pizzo (1983); they provided evidence that the half-molecule, prepared as described above, still functions as a protease inhibitor. Further evidence for the integrity of the half-molecule is provided by the ability, when excluding the iodoacetamide step, to reassociate half-molecules, prepared by DTT-treatment of $\alpha_2 M$, to tetramers which show the SAXS curve characteristic for native unreduced tetramers.

All samples used for the SAXS and SANS measurements showed only one band on polyacrylamide gels. Unlike Gonias and Pizzo (1983) we could, however, see a time-dependent formation of at least three additional bands with less mobility than the half-molecule, when solutions were allowed to stand for a week or more. The reason for this might be the tenfold higher concentration in our experiments compared with those of Gonias and Pizzo (1983). The formation of these new bands could also be monitored with the SAXS and SANS methods as an increase in intensity at small scattering angles. One explanation for this effect might be that some disulfide bonds may be reduced but not alkylated so that aggregates held together by disulfide bonds are formed.

In order to avoid interference by these aggregates all SAXS and SANS data have been collected using freshly prepared samples. Furthermore, it was found that a low temperatures decreases the rate of aggregate formation so the SANS measurements were performed at + 5 °C and the SAXS measurements on supercooled solutions at − 10 °C.

From the primary sequence of $\alpha_2 M$ a molecular weight of about 360,000 is calculated for the half-molecule, including the carbohydrate (Sottrup-Jensen et al. 1984). The value obtained in this work is about 8% higher, 390,000. The main source of error in the molecular weight determination is the uncertainty in the concentration. In this work the concentration determination is based on the value $A^{1\%}_{1\,\mathrm{cm}} = 9.1$ for $\alpha_2 M$ (Dunn and Spiro 1967). In the literature there is, however, a variation of this figure of at least ± 10% (Hall and Roberts 1978) and the same uncertainty will directly be transferred to the molecular weight; cf. Eq. (2); in order to perform better molecular weight determinations more accurate concentration values are required.

In this work a "concentration-independent" determination of the specific volume has been performed by using the $I(0)$ values obtained by SAXS and SANS. The results obtained at + 5 °C, 0.73 cm^3/g, recalculated to 21 °C using the value $dv_2/dt = 0.00055$ cm^3/gK obtained for $\alpha_2 M$ (Sjöberg and Pap 1985), is 0.74 cm^3/g. This value is in agreement with that calculated from the composition, 0.73 cm^3/g, and with experimental values obtained for $\alpha_2 M$, 0.731 cm^3/g (Hall and Roberts 1978), and 0.735 cm^3/g (Sjöberg and Pap 1985). The error in the present determination of v_2 is difficult to estimate due to the desmearing procedure involved in the SAXS data. It can, however, be concluded from Eqs. (3) and (4) that v_2 is rather insensitive to errors in the $I(0)$ values; ± 5% errors in $I_{\mathrm{SANS}}(0)$ and $I_{\mathrm{SAXS}}(0)$ will correspond to ± 1.4% error in v_2.

The positive value of α in Eq. (1), indicating a higher scattering density at large distances from the centre of the particle, is what could be expected from the normal configuration of a protein since hydrophilic groups have, on the average, higher scattering density than hydrophobic groups (Jacrot 1976). The positive α might also reflect the localisation of the carbohydrate units which have a slight higher scattering density compared with hydrophobic amino acids.

The solid line in Fig. 1 was calculated using Eq. (1) together with the values of R_c, α and β given in Table 2. The value of β is difficult to determine as we have the lowest contrast and the largest errors at the end points of the plot. The broken line in Fig. 1 was calculated assuming $\beta = 0$; within the standard deviations it might also explain the experimental result. But, on the other hand, we cannot exclude the possibility of a positive β, different from zero. A positive β means that the centre of gravity of the scattering density does not coincide with that of the volume. Since the total $\alpha_2 M$ molecule has an α value different from zero (Sjöberg et al., to be published) we can also expect a fragment of $\alpha_2 M$ to have a positive β. However, we cannot determine if this is caused by an unsymmetric distribution of polar and non-polar groups, a non-homogeneous deuteration, or an unsymmetric distribution of the hydration shell.

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