Unusual ultrastructure of complement-component-C4b-binding protein of human complement by synchrotron X-ray scattering and hydrodynamic analysis

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Solution X-ray-scattering experiments with the use of synchrotron radiation on the human complement-component-C4b-binding protein showed that its R_G is 13 nm and that its M_r is 550000. From the known primary amino acid sequence and estimated carbohydrate content, C4b-binding protein is inferred to have a total of 7.4 ± 1 subunits. Heptameric computer models for C4b-binding protein were based on the X-ray-scattering curve to a resolution of 6.4 nm, and literature values for sedimentation coefficients and electron-microscopy images. The macromolecule was represented by a bundle of seven arms held together at the C-terminal end and spaced out by a base containing 23% of C4b-binding protein by volume. If the overall length of each arm is assumed to be 33 nm as seen in electron microscopy, the solution data indicate an average arm-axis angle of 5–10°. The seven arms of C4b-binding protein are found to be close together, in distinction to the splayed-out images seen in electron micrographs.

INTRODUCTION

The complement-component-C4b-binding protein (C4BP) is a glycoprotein component of the complement cascade of immune defence that participates in the classical pathway of complement activation (for reviews see Reid & Porter, 1981; Reid, 1983; Lachmann & Hughes-Jones, 1984). On activation of the first component of complement C1 to C1i, the subunit C1s within C1 cleaves components C2 and C4 into C2a and C2b and into C4a and C4b respectively. The C3 convertase is formed from the complex of C4b with C2a. C4BP binds to C4b and acts as a cofactor in the degradation of C4b into a mixture of C4c and C4d by the enzymic action of Factor I. Thus C4BP controls the amount of the C3 convertase C4b2a that is present in serum.

The proteins of the complement cascade often exhibit unusual structural properties, and C4BP is no exception. A knowledge of the overall structural properties of C4BP is essential to an appreciation of how C4BP is able to effect control of C4b. Sedimentation coefficients and frictional ratios indicate a highly elongated structure (Scharfstein *et al.*, 1978; Villiers *et al.*, 1981; Dahlbäck, 1983). The ultrastructure of the molecule has been shown by electron microscopy to consist of seven extended arms fixed together at one end (Dahlbäck *et al.*, 1983; Dahlbäck & Müller-Eberhard, 1984). Solution X-ray scattering and the construction of computer models for the scattering curve is a technique that enables low-resolution structural models for C4BP to be evaluated and tested (Perkins & Weiss, 1983; Perkins, 1985), even though the technique will not lead to a unique structure. In the present study this methodology is applied to C4BP to show that it can be represented as a

seven-subunit structure with lengths that are as predicted from electron microscopy. Its solution structure is, however, more compact than expected from electron-microscopy images. The biological significance of this structure is discussed in terms of the known primary sequence of C4BP and the interaction of C4BP and C4b and Factor I.

MATERIALS AND METHODS

C4BP was prepared by following the procedure described in Chung *et al.* (1985a). To avoid the non-specific aggregation of C4BP that was observed in preliminary work, the purified C4BP was gel-filtered on a 150 ml Sephacryl S1000 column run at 6 °C with a high-salt buffer (250 mM-NaCl/50 mM-6-aminohexanoic acid/5 mM-EDTA/50 mM-Tris/HCl, pH 7.4). Two column fractions from this preparation were measured directly by X-ray scattering (0.18 mg/ml and 0.32 mg/ml). Higher C4BP concentrations (0.64 mg/ml and 1.45 mg/ml) were obtained by the use of Minicon B15 concentrators on pooled sample fractions at 6 °C, and were measured by X-ray scattering within the hour after concentration. Samples were assayed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after the X-ray experiments and showed no degradation.

Sample concentrations were derived from the absorption at 280 nm for use in calculations of M_r (see below). Absorption coefficients were calculated (Table 1) from amino acid sequence data that are available for C4BP as well as for Clq, C3, C4 and Factor B of complement, α_2 -macroglobulin and α_1 -acid glycoprotein. If the absorption coefficient of C4 is taken as 9.15, the mean of the two literature values, it is seen from Table 1 that in

Abbreviation used: C4BP, complement-component-C4b-binding protein.

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Table 1. Comparison of calculated and literature absorption coefficients

C1q, C3, C4 and Factor B are components of the complement cascade. Amino acid sequences were taken from the following sources: C1q (Reid, 1979; Reid *et al.*, 1982; K. B. M. Reid, unpublished work); C3 (de Bruijn & Fey, 1985); C4 (Belt *et al.*, 1984); Factor B (Gagnon & Christie, 1983; Morley & Campbell, 1984; Mole *et al.*, 1984); C4BP (Chung *et al.*, 1985b); α_2 -macroglobulin (Sottrup-Jensen *et al.*, 1984); α_1 -acid glycoprotein (Schmid *et al.*, 1973). M_r values were calculated from the amino acid sequence and known or estimated carbohydrate contents (Dunn & Spiro, 1967b; Yoshima *et al.*, 1981; Perkins *et al.*, 1984; S. J. Perkins & R. B. Sim, unpublished work; Chan & Atkinson, 1985; Table 2 of the present paper). Calculated absorption coefficients, $A_{280}^{1\% \cdot 1 \text{ cm}}$, were obtained from molar absorption coefficients ϵ_M^0 by using $A_{280}^{1\% \cdot 1 \text{ cm}} = 10 \epsilon_M^0 / M_r$, where $\epsilon_M^0 = 5500 \Sigma \text{Trp} + 1340 \Sigma \text{Tyr} + 150 \Sigma \text{Cys}$ (Wetlaufer, 1962). Literature absorption coefficients were taken as follows: C1q (Reid *et al.*, 1972); C3 (Tack & Prahl, 1976); C4 (Nagasawa & Stroud, 1977; Reboul *et al.*, 1980); Factor B (Curman *et al.*, 1977); C4BP (Villiers *et al.*, 1981); α_2 -macroglobulin (Schultze & Heremans, 1966; Dunn & Spiro, 1967a; Sober, 1970; Hall & Roberts, 1978); α_1 -acid glycoprotein (Schmid, 1953).

Serum glycoprotein	Total of 280 nm-absorbing residue			M_r	$A_{280}^{1\% \cdot 1 \text{ cm}}$	
	Trp	Tyr	Cys		Calculated	Literature
C1q	18	138	66	457000	6.43	6.82
C3	17	57	27	193000	9.01	9.7
C4	18	51	28	197000	8.70	8.3, 10.0
Factor B	13	33	23	94000	12.54	12.7
C4BP subunit	10	25	36	70500	13.32	9.3
α_2 -Macroglobulin	44	224	96	713000	7.80	8.1, 9.07
α_1 -Acid glycoprotein	3	11	4.5	37300	8.55	8.4, 8.93

six cases out of seven the literature absorption coefficients uniformly overestimated the calculated values by a mean factor of 1.06 ± 0.03 , which is as expected (Wetlaufer, 1962). The exception is C4BP, where the literature value is 30% less than the calculated value. Thus, for this present study, the absorption coefficient of C4BP is taken as 14.1 on the basis of the calculated value (Table 1) and the above factor of 1.06. For Factor H (M_r 155000), the absorption coefficient is taken as 14.2 (Sim & DiScipio, 1982).

Solution X-ray-scattering experiments were performed on the synchrotron radiation source operating at 2 GeV at currents between 100 and 250 mA at the S.E.R.C. facility at Daresbury, with the camera at station 7.3 (Nave *et al.*, 1985). The sample-to-detector distance was measured as 2.185 m and confirmed with wet stretched rat-tail collagen (spacing 67 nm) and dry white adductor muscle from the North American clam *Mercenaria mercenaria* (spacing 14.4 nm) as standards. The wavelength, λ , was 0.1608 nm. The linear detector thus gave an effective range of scattering vectors Q of 0.075–0.979 nm⁻¹, where $Q = 4\pi \sin \theta / \lambda$ and 2θ is the scattering angle. X-ray samples were measured at 6 °C in alternation with the buffer backgrounds. Sample counting times ranged between 5 and 13 min. Data reduction employed the program SWANAL, which involved subtraction of the buffer background from the sample and correction for the detector response (measured with a uniform radioactive source).

The radius of gyration, R_G , and the forward scattering at zero angle $I_{(0)}$ were determined from the Guinier relationship (Kratky, 1963):

$$\ln I_{(Q)} = \ln I_{(0)} - R_G^2 Q^2 / 3$$

with the intensities of the scattering curves at small Q . For elongated rod-like particles, this expression is valid for $QR_G < 0.7$ and becomes more approximate at larger Q values. Since in this work the scattering curves were

measured in a QR_G range of 1–13, Guinier fits had to be derived with the use of the QR_G range 1–1.6. The validity of this approximation was tested by curve simulations (see the Results section). For elongated rod-like particles, it is possible also to measure the radius of gyration of the cross-section R_{XS} and its corresponding intercept at zero Q from the relationship (Kratky, 1963):

$$\ln(I_{(Q)} \cdot Q) = \ln(I_{(Q)} \cdot Q_{(\lim Q \rightarrow 0)}) - R_{XS}^2 Q^2 / 2$$

For such elongated particles, knowledge of the R_G and R_{XS} values gives the length of the rod-like particle L from:

$$L^2 = 12 (R_G^2 - R_{XS}^2)$$

Likewise, combination of the $I_{(0)}$ and $I_{(Q)} \cdot Q_{(\lim Q \rightarrow 0)}$ values gives the length L from:

$$L = \frac{\pi I_{(0)}}{I_{(Q)} \cdot Q_{(\lim Q \rightarrow 0)}}$$

The experimental values of $I_{(0)}/c$ (where c is the sample concentration) leads to relative molecular masses, M_r , from (Kratky, 1963):

$$\frac{I_{(0)}}{c} = K \cdot M_r (z_1 - \rho_2 \bar{v}_1)^2$$

where z_1 is the number of moles of electrons/g of macromolecule, $\bar{v}_1$ is the partial specific volume of the macromolecule, and ρ_2 is the solvent electron density. K is a constant that takes into account the instrumental geometry. In this work, M_r values were determined by the use of C4u of human complement as a standard (Table 2). This was measured in the same buffer as used for C4BP; calculations showed that $\bar{v}_1$ is similar for C4u and C4BP (Table 2). Thus the squared term above becomes constant also.

Modelling calculations of the X-ray-scattering curves, radius of gyration, R_G , and sedimentation coefficient, $s_{20, w}^0$, were carried out as described elsewhere (Perkins &

Weiss, 1983; Perkins, 1985). The macromolecule was represented as 602 cubes of side 1 nm whose total volume corresponded to that of the macromolecule. The scattering curves were simulated (Rolbin *et al.*, 1973) by the use of overlapping Debye spheres positioned at the centres of the cubes, where the volume of each sphere is 1 nm³ and their diameter is thus 1.24 nm. At $Q = 1 \text{ nm}^{-1}$, the form-factor-squared term for the spheres is 0.926 of its value at zero Q , and thus the finite size of the spheres can be neglected in the plots of $\ln I$ versus Q in Fig. 4. The R_G of the 602 spheres is calculated from Damaschun *et al.* (1979) (Fig. 3):

$$R_G^2 = \sum_{k=1}^n n^{-1} (R_k^2 + r_k^2)$$

where R_k is the R_G of a sphere k , and r_k is the distance between the sphere and the centre of the macromolecule. Hydrodynamic frictional coefficients were calculated from the modified Oseen tensor procedure of Bloomfield (Garcia de la Torre & Bloomfield, 1977*a, b*; Perkins, 1985). For these calculations, the Debye model of C4BP is converted into its hydrodynamic equivalence by the use of non-overlapping spheres in tangential contact (Fig. 2), whose overall length reproduced that of the Debye model and whose overall volume is that of the macromolecule. The expansion in thickness of the arms makes some allowance for hydration (Perkins, 1985). The amino acid composition was derived from the DNA sequence data

of Chung *et al.* (1985*b*). The carbohydrate composition was estimated (Table 2). Protein and carbohydrate volumes were calculated from Chothia (1975) and Perkins *et al.* (1981).

RESULTS

Experimental X-ray-scattering curves

X-ray-scattering curves were measured for nine samples of C4BP at four concentrations between 0.18 and 1.45 mg/ml in two main-beam sessions on the X-ray solution camera (Nave *et al.*, 1985). The scattering curves showed two distinct regions (cf. Fig. 4 below) that correspond to the Guinier curve at Q less than 0.3 nm^{-1} and the wide-angle curve at Q beyond 0.3 nm^{-1} . On a preliminary hypothesis that C4BP is a rod-like particle in solution, these regions were analysed respectively by using Guinier plots of $\ln I$ versus Q^2 to obtain the R_G and $I_{(0)}$ values of C4BP, and cross-sectional plots of $\ln(I \cdot Q)$ versus Q^2 for the corresponding cross-sectional R_{XS} and $I_{(0)}$ parameters (see the Materials and methods section). The R_G values were obtained in a range of QR_G of 1–1.6 that is larger than that ordinarily used in Guinier fits for reason of the large beam-stop in use. A sufficiently accurate R_G measurement requires as condition that $QD \leq \pi$, where D is the maximum dimension of the particle (Pilz *et al.*, 1979). Since D can be estimated as 33–36 nm from electron microscopy, Q has to be

Fig. 1. Guinier analyses of the C4BP scattering curves

(a) Summary of the $I_{(0)}/c$ and apparent $I_{(0)} \cdot Q_{(\lim Q \rightarrow 0)}/c$ values for C4BP as a function of the concentration c and a comparison with the $I_{(0)}/c$ values for C4u, which were measured concurrently. Error bars here and in (b) correspond to the counting statistics. The vertical scale is the same in both sections. (b) Summary of the R_G and apparent R_{XS} values for C4BP as a function of sample concentrations.

Table 2. Composition data on a monomer of C4BP and on C4u

	Intact C4BP monomer	C-Terminal core of C4BP	N-Terminal arms of C4BP	C4u
Residues				
Amino acids	549	154	395	1711
Carbohydrate*	33–51	22–34	11–17	47
Volume (nm ³)				
Protein	77	22	55	240
Carbohydrate	7–11	5–8	2–4	10
Total	86	28	58	250
M_r				
Protein	61 500	17 400	44 100	188 200
Carbohydrate	6800–11 000	4600–7300	2300–3700	9000
Total	70 500	23 400	47 100	197 000
Partial specific volume, $\bar{v}$ (ml/g)				
Total	0.740	0.729	0.745	0.764

* Four potential carbohydrate sites that have been identified for C4BP from the DNA sequence, of which three are glycosylated. Ranges of M_r values and volumes are given to correspond to the extremes of biantennary or tetra-antennary carbohydrate types, and mean values are assumed for the total glycoprotein. For C4BP, the carbohydrate composition data given in Reid & Gagnon (1982) suggest 2–3 glycosylation sites; the data of Nagasawa & Stroud (1980) suggest 1.3–2.2 glycosylation sites. The carbohydrate content of C4u is taken from Chan & Atkinson (1985), where four attachment sites have been identified out of a total of six potential sites.

0.09 nm⁻¹ or less; this is compatible with the experimental Q range of 0.075 nm⁻¹ upwards. In addition, test calculations using the curve fit of Fig. 4(a) below showed diminutions of only 4–5% in the R_G and $I_{(0)}$ values in the QR_G range 1–1.6, which can thus be neglected. The cross-sectional parameters were obtained in a Q range 0.38–0.54 nm⁻¹ in order to use the intensities measured at the lower C4BP concentrations while minimizing the effect of background noise at larger Q . The results are summarized in Fig. 1. Despite less-satisfactory counting statistics at the lower concentrations, analyses showed no concentration-dependence of either the shape or intensity parameters in either the Guinier or cross-sectional analyses (Fig. 1). This is further substantiated by the comparison of the calculated and experimental scattering curves with C4BP at 0.32, 0.64 and 1.45 mg/ml below (Fig. 4). The distance between individual C4BP molecules can be calculated as 166 nm at 0.18 mg/ml, decreasing to 83 nm at 1.45 mg/ml. Since electron microscopy has reported the length of C4BP to be at least 33 nm (Dahlbäck *et al.*, 1983), this sets a lower limit to the long dimension of C4BP as 33 nm. Too high a C4BP concentration will increase the proximity of the macromolecules, and the risk of non-specific aggregation or interparticle interference phenomena.

M_r calculations were made for C4BP by using the $I_{(0)}/c$ values for C4BP referenced to that of C4u as a known M_r standard (Table 2). Data for C4u were measured in four concentrations between 1.8 and 4.4 mg/ml in the same buffer as that for C4BP during one of the two main-beam sessions (Fig. 1). The ratios of $I_{(0)}/c$ values lead to an M_r of 520 000 $\pm$ 60 000 for C4BP. Since the primary sequence of a C4BP monomeric unit (including the carbohydrate content) (Chung *et al.*, 1985b) gives an M_r of 70 500, the average total of subunits in C4BP is 7.4 $\pm$ 0.9. A second determination of the M_r of C4BP was made with Factor H as the reference, where the ratio of $I_{(0)}/c$ values now gives an M_r of

560 000 $\pm$ 70 000, which corresponds to an average of 7.9 $\pm$ 1.0 subunits per molecule. A third determination of M_r is derived below from a comparison of the Guinier and cross-sectional intensities at zero Q , and this gave a total of 7.3 $\pm$ 1.0 arms, which leads to an M_r of 510 000 $\pm$ 70 000. The present estimates of M_r are comparable with other values of 450 000 (Nagasawa & Stroud, 1980), 540 000 and 590 000 (Scharfstein *et al.*, 1978), 590 000 and 660 000 (Villiers *et al.*, 1981) and 570 000 (Dahlbäck, 1983). By using these published values, the total of subunits of M_r 70 500 thus ranges between 6.4 and 9.4 per molecule. The direct observation of C4BP by electron microscopy and analyses by limited proteolysis with chymotrypsin suggest seven subunits (Dahlbäck *et al.*, 1983; Dahlbäck & Müller-Eberhard, 1984). C4BP exists in two forms that differ by about 50 000–70 000 in M_r (Scharfstein *et al.*, 1978; Villiers *et al.*, 1981). The present X-ray data are compatible with a seven-subunit structure that coexists with an equal amount of an eight-subunit structure as suggested by sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analyses in non-reducing conditions. An alternative possibility of a mixture of six-subunit and seven-subunit assemblies from the analyses below is not ruled out. Although the existence of different forms has been attributed to possible complex-formation with protein S in the C4BP preparation [see Dahlbäck (1983) for a discussion], a significant amount of protein S could not be identified in the samples used in this present work (Chung *et al.*, 1985a).

The scattering data are first considered on the hypothesis that C4BP is a rod-like particle. The apparent length of C4BP can be calculated to be 44 nm from combination of the R_G of 13 nm and the R_{XS} of 2.25 nm (see the Materials and methods section). The use of the corresponding $I_{(0)}/c$ values of 180 and 0.29 (Fig. 1) gives, however, an apparent length of 195 nm. The 4–5 fold discrepancy in the result of the two calculations shows

Fig. 2. Computer models for the low-resolution structure of C4BP

(a) Subunit of C4BP used in model A for C4BP, with spheres of 1.0 nm in diameter. This shows the chymotryptic cleavage site determined from the primary sequence (Chung *et al.*, 1985b) and the arm-axis angle. (b) Hydrodynamic model of C4BP, where each arm was modelled with 17 spheres of diameter 1.95 nm, and the base was represented by a sphere of diameter 6.44 nm. The arm-axis angle is 10° . (c) Scattering model (A) for C4BP, in which the arm-axis angles are 10° , 10° , 10° , 5° , 15° , 15° and 5° in that sequence. (d) Scattering model (B) for C4BP, in which the arm-axis angles are 7° , 7° , 2° , 12° , 2° , 7° and 12° in that sequence. A hydrodynamic model for C4 is shown in (b) (see the Discussion section).

that C4BP cannot be approximated as a rod-like particle in solution and that its shape is much more complex. This discrepancy is examined further in control calculations. Hence the cross-sectional parameters given in Fig. 1 have been useful only as an empirical monitor of the wide-angle curve, as used above.

Computer modelling of C4BP

With regard to the M_r and subunit estimates above, C4BP was modelled as a seven-subunit structure. From

electron microscopy, the arms were taken to be of length 33 nm; after chymotryptic digestion, the isolated arms (Fig. 2) were seen to be 29 nm in length, and the base was observed to be of diameter 6 nm with the arm stubs of length 4 nm (Dahlbäck *et al.*, 1983; Dahlbäck & Müller-Eberhard, 1984). The primary sequence of C4BP indicates that the arms are not collagenous in structure (Chung *et al.*, 1985a). Since collagenous structures would be prone to shrinkage on dehydration in the electron microscope [which has been suggested for C1q in Perkins

Fig. 3. Dependence of the R_G values and $s_{20,w}^0$ values for C4BP on the arm-axis angle of C4BP

The scattering model A and the hydrodynamic model that were used in these simulations are depicted in Fig. 2. The broken lines correspond to the experimental values of R_G (text) and the sedimentation coefficient $s_{20,w}^0$ (Scharfstein *et al.*, 1978; Villiers *et al.*, 1981).

(1985)], these dimensions appear reasonable to use in the modelling of C4BP.

Sphere models were developed on the basis of the arm length of 33 nm and a series of different arrangements of the spheres at the base of C4BP (Fig. 2). The chymotryptic cleavage point is known from the primary sequence to be at Tyr-395 (Chung *et al.*, 1985b), and thus summations of the residue volumes gave a total of 58 nm³ (Table 1; Chothia, 1975; Perkins *et al.*, 1981). It was therefore convenient to model the outer arm as a 29 × 2 × 1 array of spheres, each of volume 1 nm³, and overall dimensions 29 nm × 2 nm × 1 nm. One sphere corresponds to just under seven amino acid or carbohydrate residues. The diameter of 3 nm from electron microscopy leads to a volume of 205 nm³ for the outer arm, which is too large by a factor of 3.5-fold (Dahlbäck *et al.*, 1983). The inner-arm segment of length 4 nm was modelled by using a 4 × 2 × 1 array of spheres, thus leaving 20 spheres per C4BP subunit for arrangement in the base of C4BP. Since α -helical structures for the C-terminal 58 amino acids appear favourable by Chou-Fasman predictive methods (Chung *et al.*, 1985a), a compact array of 5 × 4 × 1 spheres was employed (Fig. 2) to approach the concept of a globular shape. These Debye spheres were used first to calculate the R_G of the assembly of spheres as a function of the arm-axis angle (Fig. 3) for comparison with the experimental R_G of 13 nm (Fig. 1). Complementary calculations also shown in Fig. 3 were carried out to calculate the sedimentation coefficient, $s_{20,w}^0$, from a hydrodynamic model of C4BP for comparison with literature values of $s_{20,w}^0$ of 10.7 and

11.2S (Scharfstein *et al.*, 1978; Villiers *et al.*, 1981). The hydrodynamic model was constructed from seven arms, each composed of 17 spheres of diameter 1.95 nm and total length 33 nm, and a single sphere of diameter 6.44 nm to represent a globular base. The arms are in tangential contact with the equator of the large sphere (Fig. 2).

As the arm-axis angle is increased from 0° to 90° in the C4BP models, Fig. 3 shows that the R_G rises from 12.3 nm to 21.2 nm, whereas the $s_{20,w}^0$ decreases from 12.1 to 6.7 S. The intersection of the experimentally obtained data with the calculated curves shows that both physical parameters correspond to the same arm-axis angle of 7°. This indicates that the experimental R_G and $s_{20,w}^0$ values are self-consistent.

The next stage of modelling calculations compared the simulated scattering curves with the experimental scattering curves in the Q range 0.08–0.98 nm⁻¹ (Fig. 4). Comparison with a curve simulation for a cylindrical rod assembled from small spheres (whose R_G and volume are the same as those for C4BP) showed large deviations beyond $Q = 0.2$ nm⁻¹ (Fig. 4). Further calculations in which this rod of axial proportions 11:1:1 is replaced by a triaxial object with axes in the proportions 43:12:1 showed improved agreement with experiment (Fig. 4). Although a simulation with a triaxial object with axial proportions 67:36:1 comes close to the experimental C4BP scattering curve (not shown), this structure has a thickness of only 0.66 nm, which is not only physically implausible but also is in conflict with the seven-arm substructure seen by electron microscopy. Accordingly an explanation of the experimental curve was derived in terms of the C4BP subunit shown in Fig. 2(a), which was arranged in a symmetrical seven-subunit structure with the arm-axis angle set to 10° as predicted from the R_G and $s_{20,w}^0$ simulations. This gave a promising curve fit. Larger arm-axis angles readily lead to increasing discrepancies in the Gaussian section of the scattering curve below $Q = 0.3$ nm⁻¹, thus providing further confirmation that the R_G of C4BP is 13 nm and that the arm-axis angle is 10°. The final model, which gave the good curve fit of Fig. 4 (model A), was obtained on the assumption that some flexibility of $\pm 5^\circ$ in the arm-axis angle was allowed. While the mean angle was kept at 10°, four angles were re-set to 5° and 15° (Fig. 2c). A second type of model was now developed to show that a sevenfold symmetry for the base of C4BP is not a requisite for a good curve fit. Model B of Fig. 2(d) had a compact base that was created from a total of 140 spheres in a rectangular array of dimensions 10 × 10 × 2 from which the four corners and an oval section of 12 spheres at the centre had been removed. A good curve fit, as shown in Fig. 4, was obtained with a mean arm-axis angle of 7°. Flexibility of $\pm 5^\circ$ was again incorporated by the use of four angles set at 2° and 12°. Model A and model B have holes of diameter 1.3 nm and 3.1 nm respectively in the centre of their bases, in accord with a diameter of 1.3 nm seen by electron microscopy (Dahlbäck & Müller-Eberhard, 1984). The closest radial distance, r , between the C4BP central axis and the surface of the arm (see Fig. 2a) is 3.7 nm in model A and 4.8 nm in model B. In conclusion, the use of model A and model B leads to equivalent curve fits, and illustrate the types of models that are allowed by the scattering data to a structural resolution ($2\pi/Q$) of 6.4 nm as postulated from electron microscopy.

Fig. 4. Curve fits for the synchrotron X-ray scattering curves of C4BP in terms of models A and B for C4BP as shown in Fig. 2

The upper curve fits out to $Q = 1 \text{ nm}^{-1}$ were based on models A and B. The lower curve fits out to $Q = 0.4 \text{ nm}^{-1}$ were based on model A in two dilutions. The additional broken line shown in the fit for model A is the curve simulation based on a rod-like particle with volume and R_G identical with that of the C4BP model and axes in the proportions 11:1:1. The simulation based on a triaxial object with axial proportions 43:12:1 is shown also (see the text).

Calculations were next made with models for C4BP where the model had one to ten subunits (Fig. 2a) and unequal arm-axis angles of mean value 10° and varying in a range of $\pm 5^\circ$. These were done to investigate the discrepancy in the length calculation for C4BP found on combination of the experimental Guinier and cross-sectional analyses. For a single arm and base, the two

values of L calculated from the R_G and from the $I_{(0)}$ parameters are similar and slightly greater than the length of 37 nm in this model. Increasing the number of arms to ten causes the apparent value of L calculated from R_G and R_{XS} to increase to 47 nm. That value for L calculated from $I_{(0)}$ and $QI_{(0)}(\lim_{Q \rightarrow 0})$ increased more rapidly and approximately equals the product of the total of

subunits and the length of the subunit of 37 nm. The globular base of C4BP contributes 23% of the total volume. Further calculations based on the removal of the base improve these correlations in terms of overall length calculations. In conclusion, the scattering curve of C4BP reflects the sum total of subunits in the Guinier region and approximately resembles the scattering curve of a single subunit at large Q . This accounts for the experimental discrepancy in the length determinations. The simulations show furthermore that the total of subunits can be estimated from the $I_{(0)}$ and $QI_{(0)}(\lim_{Q \rightarrow 0})$ values if the length of a C4BP subunit is taken to be 37 nm, and if a Q range of $0.53\text{--}0.97\text{ nm}^{-1}$ (Fig. 4) is employed in cross-sectional analyses. Use of the data at 1.45 mg/ml and 0.64 mg/ml concentrations gave L values of $270 \pm 40\text{ nm}$, which correspond to 7.3 ± 1.0 subunits and an M_r of 510000 ± 70000 .

Final control calculations were carried out in order to explore the assumptions used in models A and B for C4BP. These relate to (i) the number of arms and (ii) their length, and to (iii) the shape of the base and (iv) the possible binding of protein S. (i) Models with six to ten subunits were tested further. For an arm-axis angle of 10° , the calculated R_G showed only a slight increase of 0.1 nm per added arm, and was thus insensitive to the total of subunits. Models with six arms or eight arms gave fits within error of the experimental curves of Fig. 4, but nine-arm and ten-arm models gave calculated intensities that were too low at large Q . This is in agreement with the calculations above of the C4BP M_r , where values corresponding to 7.3–7.9 arms had been obtained. (ii) Arm lengths that are longer than 36 nm or shorter than 18 nm give R_G and $s_{20,w}^0$ values that are outside the range of the experimental R_G and $s_{20,w}^0$ values. Arm lengths of 29 nm and 25 nm correspond to average arm-axis angles of 20° and 30° respectively. Hence a $\pm 10\%$ error in the arm length implies an inverse error of $\pm 10^\circ$ in the arm-axis angle. (iii) Calculations were carried out to examine the base structures for C4BP that are allowed by the scattering curve. In place of the array of 5×4 spheres shown in Fig. 2(a), arrays of 2×10 , 3×7 , 4×5 , 6×3 , 7×3 and 10×2 spheres were tested with seven-subunit structures similar to model A. The first three led to comparable R_G values with model A for an arm-axis angle of 10° ; however, the calculated intensities beyond $QR_G = 2$ were greater than the experimental intensities (Fig. 4). The last three gave R_G values that were too large and calculated intensities that were below the experimental curve of Fig. 4. The corresponding calculations for model B replaced the array of $10 \times 10 \times 2$ spheres with others of dimensions $9 \times 8 \times 3$, $7 \times 7 \times 4$ and $6 \times 7 \times 5$. Although comparable R_G values could be obtained in all cases, the calculated intensities were too high beyond $QR_G = 2$, with the largest difference being found for the most compact array. (iv) Final calculations to investigate the possible binding of Protein S (M_r 70000) to the base of C4BP (Dahlbäck *et al.*, 1983) evaluated the addition of an extra 70–104 spheres at this position. Although these changes lead to M_r increases of 12–17%, increases in the R_G values and deviations between the experimental and calculated scattering curves, it was not clear if the binding of Protein S could be unequivocally identified.

The results of these control calculations show that C4BP has 7 ± 1 arms and an arm-axis angle of $7 \pm 10^\circ$. Only a limited range of structures for the C4BP base are able to satisfy the experimental curves.

DISCUSSION

This work has shown that X-ray synchrotron radiation can be successfully applied to solutions of C4BP, despite the experimental constraints of low sample concentrations and elongated structures. The scattering-curves analyses indicate that in solution C4BP has an average arm-axis angle of $7\text{--}10^\circ$, i.e. that the arms of C4BP are close together. The central assumption behind the models is that the arms are 33 nm in length (Dahlbäck *et al.*, 1983; Dahlbäck & Müller-Eberhard, 1984). The main difference between models A and B (Fig. 2) in comparison with the electron-microscopy model is that the arms of C4BP are splayed out extensively in several directions in the electron micrographs. One explanation of the difference might be that the C4BP molecule has been distorted in the course of sample preparation for electron microscopy. This would imply that the C4BP arms are flexible. Alternatively, the C4BP arms can be shorter than the length of 33 nm that has been taken from electron microscopy. In this case, the arm-axis angle has to be increased in order to preserve the agreement between the calculated and experimental R_G and $s_{20,w}^0$ values. However, angular changes of only $\pm 10\text{--}20^\circ$ appear to be involved, in consequence of the likely precision of the electron-microscopy work.

The primary structure of C4BP offers some insight into the close disposition of the arms that are predicted by models A and B. The 395 amino acids of the *N*-terminal outer arm bear 39 aspartic acid + glutamic acid residues and 40 lysine + arginine residues. Since there are also 13 histidine residues, which will have a pK of 6.0–7.4 (Elmore, 1968), the C4BP outer arm will be electrostatically almost neutral at the pH of 7.4 employed in the present work. There will be little tendency for the arms to repel one another, as has previously been discussed in the case of C1q (Perkins, 1985). Models A and B (Fig. 2) suggest furthermore that the function of the base of C4BP is to space out the C4BP arms in a circle of inner diameter 8–11 nm.

The biological significance of the C4BP structure can be appraised in terms of the structure of C4b, although little is known about the Factor I-mediated cleavage of C4b with C4BP acting as cofactor. C4 is homologous in structure to C3 (de Bruijn & Fey, 1985), where the low-resolution structure of C3 has been described by solution scattering in terms of a flat elongated ellipsoid of dimensions $18\text{ nm} \times 10\text{ nm} \times 2\text{ nm}$, and C3u and C3b are similar in structure to C3 (S. J. Perkins & R. B. Sim, unpublished work). A comparison between the structure of C4BP and that presumed for C4 is shown in Fig. 2(b). Since the thickness of C4u or C4b is expected to be of the order of 2 nm, it is therefore sterically possible to pack up to seven C4u or C4b molecules with their long axes parallel to the C4BP arms in a lengthwise fanned arrangement on the tips of the C5BP arms (Dahlbäck *et al.*, 1983) or along the length of the C4BP arms. It has been reported that up to 4.8 molecules of C4b can bind per C4BP molecule (Scharfstein *et al.*, 1978); more recently it has been suggested that there are four strong sites and two weak sites for C4b on C4BP (Ziccardi *et al.*, 1984). These considerations imply that the binding site for C4BP on C4u or C4b is close to the edge of its flat ellipsoidal shape. That about five C4b molecules bind to C4BP and not about seven suggests that the arms of C4BP are sufficiently close together to offer steric hindrance to

the full binding of C4b, in agreement with the structure for C4BP shown in Fig. 2 but not with the splayed-out structure suggested from electron microscopy. Since the absorption coefficient for C4BP is larger by this work than previously supposed (Table 1), an alternative interpretation of the less-than-full binding of C4b or C4u to C4BP is based on the use of calculated C4BP concentrations in earlier stoichiometry experiments that are too high. Allowance for this would lead to an increase in the number of C4b or C4u molecules that bind to a single C4BP molecule.

S.J.P. acknowledges a Senior Research Fellowship from the Lister Institute for Preventive Medicine, and support from the Medical Research Council and the Science and Engineering Research Council. We thank Dr. C. Nave for generous instrumental support at Daresbury.

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Received 18 February 1985/23 July 1985; accepted 7 October 1985