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The use of small-angle X-ray scattering in the study of quaternary organisation of giant proteins[1]

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Abstract

We report an investigation on the quaternary organisation of hemocyanin giant proteins, performed with continuous small-angle X-ray scattering (SAXS) at the LURE facility. The molecular weight of a 11S subunit of molluscan hemocyanin was determined from the experimental data. Structural models of this subunit, which include seven or five functional units, were also derived and correlated to the whole molecule, which has the form of a hollow cylinder.

Keywords: Small angle X-ray scattering; Proteins; Quaternary structure

1. Introduction

The definition of quaternary structures of proteins having high molecular weight is generally based on the conventional transmission electron microscopy (CTEM) technique. However, this method cannot be used exclusively because several artefacts may arise during specimen preparation such as, (i) flattening and other distortions of the protein aggregate during the negative staining procedure and when being observed, (ii) uneven distribution of the contrasting media, (iii) damage of the sample under the electron beam. Furthermore CTEM essentially gives a surface description of the object, with very poor information on its internal mass distribution.

Hemocyanins (Hcs) are giant oligomeric copper proteins involved in the dioxygen transport–storage function in the hemolymph of several invertebrate species of *Mollusca* and *Arthropoda*. Their quaternary structures show two completely different organisations, each one typical of a phylum. Arthropodan Hcs are characterised by a 16S species ($M_r \approx 450$ kDa), which is a hexamer of subunits ($M_r \approx 75$ kDa) arranged as a trigonal antiprism, containing one active site each. This species is the basic component responsible for the generation of the higher aggregation forms, namely 24S (2 × 6-mer, $M_r \approx 900$ kDa), 37S (4 × 6-mer, $M_r \approx 1800$ kDa) and 62S (8 × 6-mer, $M_r \approx 3600$ kDa) (Fig. 1A).

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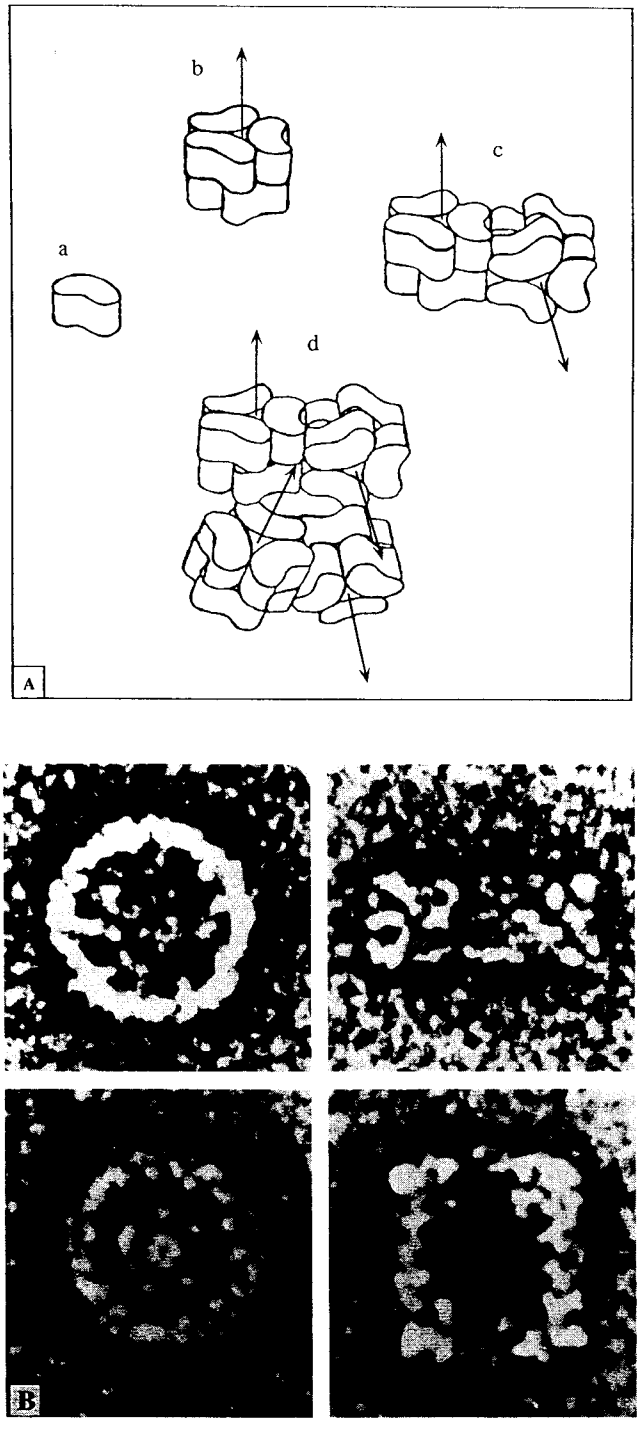

Fig. 1. (A) Arthropodan hemocyanin aggregates reconstructed on the basis of X-ray crystallography and electron microscopy: (a) subunit with M_r 75 kDa; (b) 16S 6-mer; (c) 24S 2 × 6-mer; (d) 37S 4 × 6-mer. The arrows indicate the orientation of the ternary symmetry axis [1]. (B) Electron micrographs of *Octopus* hemocyanin (upper panel) and *Rapana* hemocyanin (lower panel). The right and left images represent axial and lateral views of the molecules. Reconstruction from Ref. [1].

The subunits of molluscan Hcs consist of basic units with a 11S sedimentation coefficient ($M_r \approx 250$–450 kDa). The subunits are organised in several functional units ($M_r \approx 50$ kDa), each one containing one copper active site (see Ref. [1] and references cited therein). Aggregation of the 11S species yields specific monodispersed whole molecules, whose shape in CTEM images corresponds to that of a hollow cylinder with a quinary symmetry axis. The molecule has a diameter of 350 Å, with a length of 380 Å in gastropods and 150 Å in cephalopods. Fig. 1B shows electron micrographs of molluscan Hcs (*Octopus* upper panel; *Rapana*, lower panel). The circles (left) and rectangles (right) represent the axial and lateral views of the molecules, respectively.

We performed synchrotron radiation SAXS experiments on both molluscan and arthropodan Hcs at the LURE facility, with the aim of correlating CTEM and SAXS information on the quaternary structure of Hcs. This issue is particularly important because the 11S species is not well resolved by the CTEM technique, while the SAXS technique can provide primary information for solving protein structures.

2. Experimental

Native Hcs and dissociation products were prepared according to standard procedures [2]. Homogeneous preparations of the different protein aggregates were obtained by fast protein liquid chromatography (FPLC). A combination of gel filtration (Superdex 200 column) and ion exchange (Sepharose-Q and Mono-Q columns) chromatography was used with stripping buffer (Tris/HCl 50 mM, pH 9.2, EDTA 10 mM) to isolate subunits of arthropodan and *Octopus* Hcs, or with associating buffers (Trix/HCl 50 mM, 20 mM $CaCl_2$, pH 7.4) for the higher aggregation forms. Dissociation products of *Rapana* Hc (RHSS1, a monomeric 11S species and RHSS2, a covalent dimer) were prepared as indicated in Ref. [3].

The SAXS measurements were performed at the D24 station high-flux beamline of the LURE facility, using the standard set-up. The wavelength of the high monochromatic ($\Delta\lambda/\lambda = 10^{-3}$) X-ray

Table 1
Radii of gyration of arthropodan hemocyanins in various aggregation states (see the schematic diagram of Fig. 1A); from the gyration radius measured by SAXS on the monomer, the values of the heavier forms have been calculated by using the values of distances between the subunits, provided by X-ray diffraction and CTEM

Molecular species			R_g/Å	
No. of subunits	Species	MW/kDa	From SAXS	Calculated
1	*Carcinus m.*	75	27.3 ± 0.3	
	Limulus p.	75	28.0 ± 0.3	
6	*Carcinus m.*	450	52.6 ± 5	55.5 ± 1.5
	Limulus p.	450	58.3 ± 1.5	55.5 ± 1.5
12	*Carcinus m.*	900	70.0 ± 4	80.0 ± 5
24	*Limulus p.*	1800	107.0 ± 2	115.0 ± 4

beam was 1.488 Å. The data were collected following the high statistic procedure with a logging time $\Delta t = 400$ s. The scattered intensities were recorded by a position-sensitive proportional detector 1205 mm or 2205 mm distant from the sample to allow angular resolutions $\Delta s = 2.337 \times 10^{-4}$ Å^{-1} and $\Delta s = 1.277 \times 10^{-4}$ Å^{-1}, respectively ($s = 2\sin(\theta)/\lambda$). Further details on data collection and analysis programs are given in Ref. [4].

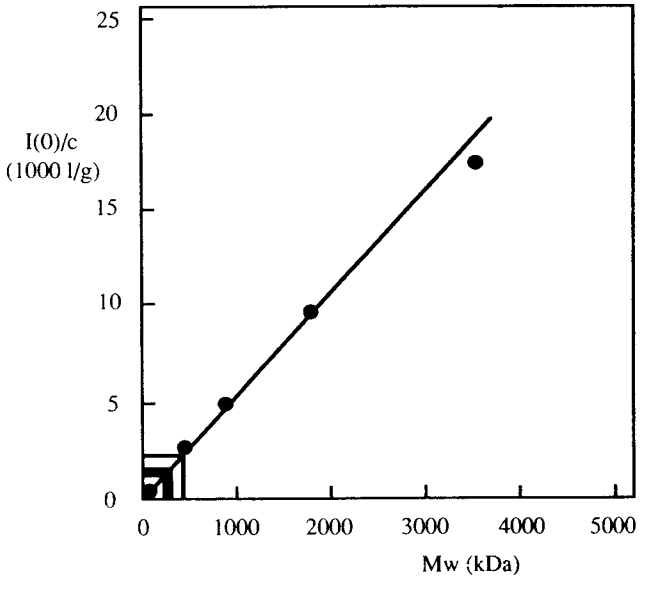

Fig. 2. Plot of zero-angle scattered intensities, normalised for concentration, versus molecular weight obtained from *Limulus poliphemus* and *Carcinus maenas* hemocyanins in various aggregation states. Dark shading indicates the confidence limit of estimated molecular weights for 11S *Octopus* and *Rapana* hemocyanins (lower values) and of the dimer of 11S *Rapana* hemocyanin (higher values).

3. Determination of molecular weight of the molluscan Hc 11S subunit

We used arthropodan Hcs as the calibration system, because their structure and M_r are well known from ultra-centrifugation, X-ray crystallography and electron microscopy (Fig. 1A) methods [1]. Solutions of Hcs from the arthropods *Carcinus maenas* and *Limulus poliphemus* in various aggregation states and at different concentrations were examined in order to evaluate the zero-angle scattered intensities and correlate them to the known molecular weights. We checked the correspondence found by calculating the gyration radius of the multimeric species on the basis of the distance distribution derived from the structure determined by

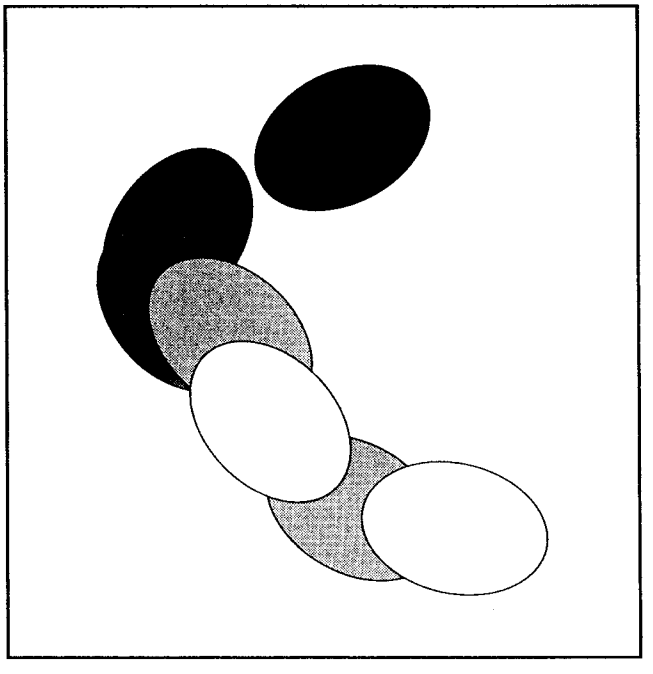

Fig. 3. Schematic representation of the eptameric model seen from the axial projection. The horizontal semiaxes of each ellipsoid measure 35 and 25 Å; the third axes measure 20 Å and have a different orientation to the horizontal plane. The various intensities of grey shading reflect the different positions of the domains located below the plane containing the first two ellipsoids (white).

X-ray diffraction and CTEM [1] (see Table 1). The values in the table agree with those measured by SAXS. We thus obtain the linear calibration curve of Fig. 2.

We used the calibration curve to determine the molecular weight of the main subunit, 11S, of molluscan Hcs (*Octopus vulgaris* and *Rapana thomasiana*). From the zero-angle scattered intensities of dissociated samples we obtained a molecular weight of 235 ± 35 kDa (Fig. 2). A value of 500 ± 20 kDa was found for the RHSS2 species of *Rapana* Hc. Taking into account the molecular weight of a single functional unit of the subunit (see above), the value obtained from the calibration curve is compatible with a pentameric model for the 11S species.

4. Structure of 11S subunit of molluscan Hcs

Molecular weights ranging from 380 to 450 kDa have been reported for the 11S structural subunit of molluscan Hcs. Correspondingly, models with seven or nine functional domains of 50 kDa each have been proposed [1,2]. In particular, an eptameric model has been suggested for *Octopus*, not in contrast to immuno-electron microscopy observations [5,6]. We have examined the two alternative models: eptameric and pentameric.

First we tried to verify the eptameric architecture determined from the three-dimensional (3D) density map of the whole molecules [5] with the aid of the model proposed in Ref. [6]. By using ellipsoidal domains lying horizontally and oriented to mimic the 3D density map (Fig. 3), we obtained a satisfactory simulation. However, this model does not give the same gyration radius as found experimentally (65 ± 4 Å), but a higher value of 74 Å (Fig. 4(b)). Fig. 4(a) instead shows the results of the same procedure applied to the pentameric model of Fig. 5. The crucial points in modelling are the shape and the mutual orientation of each domain. Models using spheres do not allow one to reproduce either the diameter and thickness of the whole molecule or the region of the scattering patterns at the smallest angles (data not shown), which depends on the overall shape of the scattering body [7]. However, elongated ellipsoids are

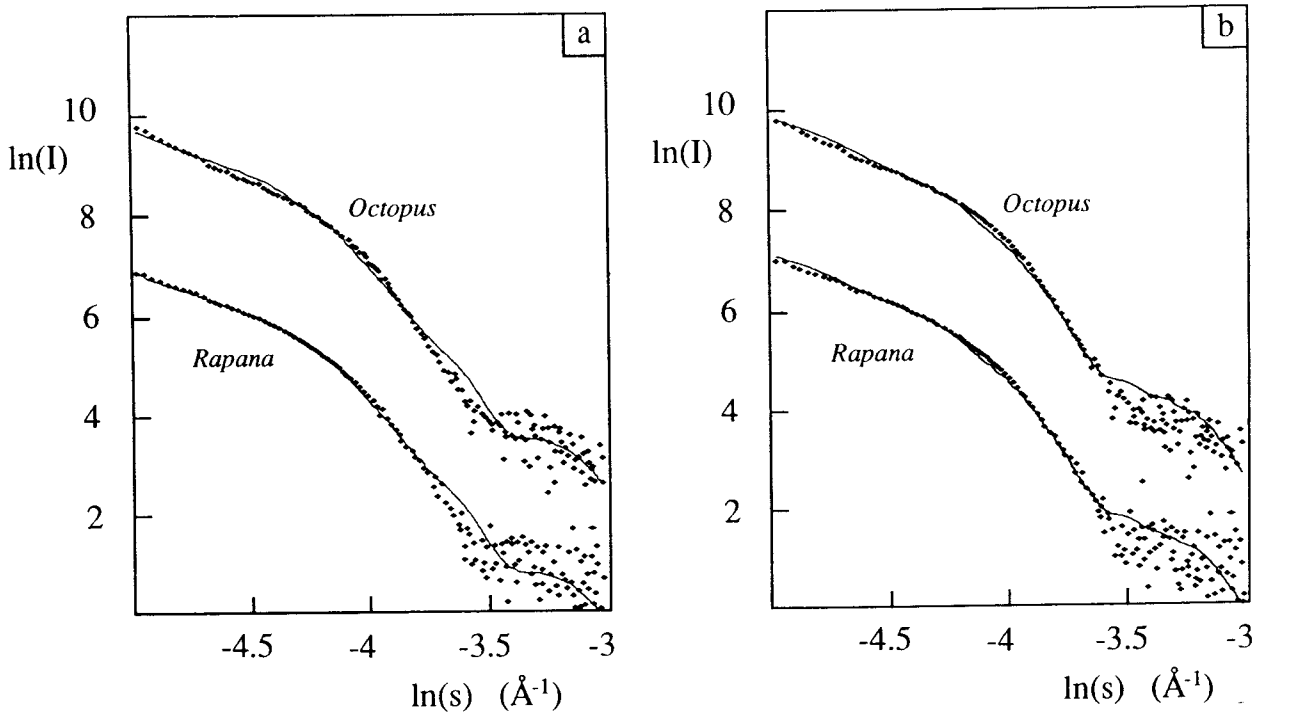

Fig. 4. Comparison of experimental scattering profiles and simulations for the pentameric (a) and the eptameric (b) models. The experimental profiles and simulations refer to 11S *Octopus* (upper curves) and *Rapana* (lower curves) hemocyanins.

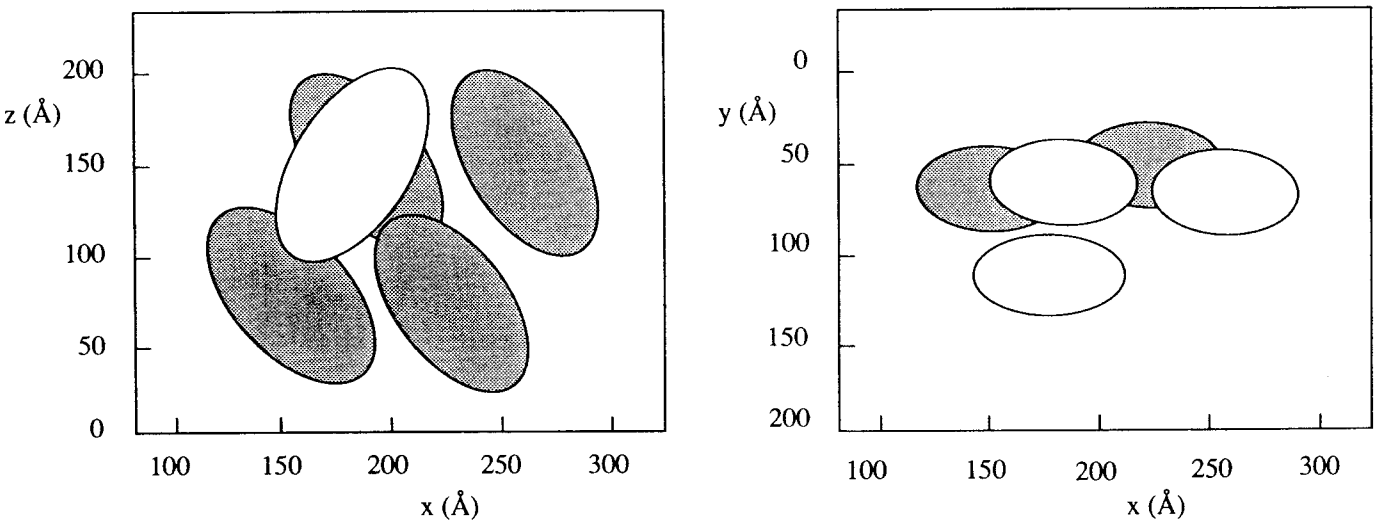

Fig. 5. Pentameric model with oblique ellipsoids seen from two different orientations. The semiaxes measure 48.5 Å, 19 Å and 26 Å. The dark ellipsoids are partially eclipsed by the white ellipsoids.

suitable for correctly fitting two tiers of subunits within the dimensions of the whole molecule of *Octopus* Hc, although a horizontal orientation of domains cannot reproduce the scattering patterns, unless their dimensions are larger than those allowed by the molecule dimensions. The quality of the simulation was improved (see Fig. 4(a)) when five obliquely oriented ellipsoids were arranged in the configuration of Fig. 5. Thus we obtained a model compatible with the accurate 3D density map of the *Octopus* whole molecule [5]. The helical form of the molecule, resulting in the helical grooves that characterise the lateral CTEM images (Fig. 1) [1,2], can also be simulated. The model reproduces both the SAXS pattern and the gyration radius of the subunit (63 Å, compared with the experimental value of 65 ± 4 Å) together with the dimensions of the whole molecule.

5. Discussion and conclusions

According to some authors, Hcs from *Octopoda* (*Octopus vulgaris*) and *Gasteropoda* (*Rapana thomasiana*) differ from each other in that their main subunits are composed of a different number of functional domains (seven or nine) [1]. If the domains are similar in shape and M_r, then the SAXS patterns given by the different subunits are also different, especially in the region of the smallest angles. In this work we show that the SAXS patterns of the 11S subunits of the two Hcs are remarkably similar. Both this fact and the values of the molecular weights given by our analysis suggest a very similar architecture. Moreover, the simulations reported here have the same validity when compared to the experimental scattering profiles given by both *Octopus* and *Rapana* Hcs. The results obtained in our study do not allow one to discriminate between an eptameric and a pentameric model. Because ellipsoids of different dimensions were used in the two configurations, a study of the structure of the isolated functional domain obtained by proteolytic fragmentation of the 11S species would certainly help the comparison. We have recently obtained very pure preparations of 50 kDa domains by trypsin or chymotrypsin incubation of whole 11S subunits, and further studies on a single domain are planned. Furthermore, the higher angle region of the scattering intensity given by the subunit should be thoroughly examined and the models refined, to verify whether they also reproduce the undulations pertinent to the dimensions of the functional domains: the insufficient concentration of the samples examined so far has not allowed the higher angle region of the scattering curves to be detected with a sufficient signal-to-noise ratio.

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References

[1] B. Salvato and M. Beltramini, Life Chem. Rep., 8 (1990) 1.
[2] G. Preaux and C. Gielens, in R. Lontie (Ed.), Copper Proteins and Copper Enzymes, Vol. II, CRC Press, Boca Raton, 1984, p. 159.
[3] K. Idakieva, S. Severov, I. Svendsen, N. Genov, S. Stoeva, M. Beltramini, G. Tognon, P. Di Muro and B. Salvato, Comp. Biochem. Physiol., 106B (1993) 53.
[4] M. Beltramini, E. Borghi, P. Di Muro, A. Ghiretti Magaldi, A. La Monaca, M. Salvato, C. Santini and G. Tognon, J. Phys. IV Coll., C8 (Suppl. JP I), 3 (1993) 249.
[5] O. Lambert, N. Biosset, P. Penczek, J. Lamy, J. Taveau, J. Frank and J.N. Lamy, J. Mol. Biol., 238 (1994) 75.
[6] J. Lamy, C. Gielens, O. Lambert, J.C. Taveau, G. Motta, P. Loncke, N. De Geest, G. Preaux and J.N. Lamy, Arch. Biochem. Biophys., 305 (1993) 17.
[7] O. Glatter, Acta Phys. Austriaca, 36 (1972) 307.