Advanced small-angle X-ray synchrotron radiation scattering study of hemocyanin oligomers using a high-performance two-dimensional detector

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Structural parameters of lyophilized hemocyanin oligomers from *Octopus vulgaris* have been obtained by continuous small-angle X-ray scattering experiments using a high-performance area detector realized at Frascati Laboratories on an Adone wiggler beam line. The new apparatus allowed fast collection of two-dimensional diffraction patterns and showed in real-time graphic display both the presence of different scattering units with superparticles of very high molecular weight and the anisotropic effects. The results of the experiments on lyophilized half-met hemocyanin derivative have stressed the fundamental presence of the 19S fraction.

1. Introduction

We report the results of a study on lyophilized native and half-met *Octopus vulgaris* (*v.*) hemocyanin, obtained by using a SAXS (small-angle X-ray scattering) camera fitted with a drift-chamber area detector at the Frascati–Adone wiggler beam line. This new apparatus, by means of a last collection of two-dimensional diffraction patterns, shown in real-time graphic display, allows one to obtain morphological information and to detect anisotropic effects on several large-size particles such as biological macromolecules, high polymers, metals and alloys, that are usually characterized by comparing different experimental methods.

Hemocyanins are specific oxygen-carrier proteins found as extracellular oligomers of high molecular weight in the hemolymph of several species of *Mollusca* and *Arthropoda*. The oxygen-transport function of the hemocyanins is due to the binuclear-copper active site. The relationships of the physiological function of hemocyanins with their molecular properties, their structure and the role of the binuclear

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copper at the active site have been recently reviewed [1,2].

Hemocyanins are also traditionally classified according to their sedimentation coefficients and dissociation–association behaviour. There are basically three morphological classes of molluscan hemocyanins, called 102S, 57S, 49S [3–5]. The 102S represents the basic structure of the class *Gastropoda*, while the 57S and 49S represent the whole molecule of hemocyanins of *Decapoda* and *Octopoda* classes, respectively. The octopoda hemocyanins exhibit dissociation into the following fractions: 49S⇌19S⇌11S. Hence, the fraction 11S represents the fundamental minimal subunit in the molecular architecture of the octopoda class, as well as in all the molluscan hemocyanins from different species [1]. The structural analysis of organization of the hemocyanin species and the relationships of the subunit-to-whole molecule have been performed combining molecular morphological studies by different techniques like ultracentrifugation and electron microscopy, along with molecular-weight determinations [6,7]. Electron microscopy has revealed that different varieties of molluscan hemocyanins have a common morphological shape, namely a hollow cylinder. The same shape has been detected for β-hemocyanin *Helix pomatia* by small-angle X-ray scattering [8].

We have used the above high-performance camera to investigate the structural organization of the 49S species of the molluscan hemocyanins, since a detailed reconstruction of the octopoda class is still undetermined. Our results on lyophilized half-met hemocyanin derivative have shown the fragmentation of the native molecule into a smaller subunit, that we have identified as the 19S fraction, and the reassociation in superparticles of very high molecular weight. For the *Octopus v.* native hemocyanin, we have confirmed the hollow-cylinder geometrical shape, and the molecular weight, deduced from our data, compares well with the value obtained from the other authors.

2. Experimental

The small-angle X-ray diffraction apparatus at the Frascati wiggler beam line was used. It incorporates a gas drift-chamber area position-sensitive detector connected to a fast computerized data-acquisition system (0.7 MHz), with a real-time graphic display, using TDC, CAMAC, VME systems and Macintosh IIx. The drift-chamber area detector has uniform sensitivity of detection, high spatial resolution (at least 155 μm) in both dimensions, very low noise, wide dynamic range and a high count acquisition rate (2×10^{-4} cps/pix). With this setup, it is possible to measure a scattering momentum of the order $k_{min}=8\times10^{-3}$ Å^{-1}. This new apparatus has already been described in detail [9,10].

For the purpose of this study, it is important to note that the SAXS camera was situated at 35 m from the wiggler source. A circularly collimated primary beam of diameter 0.5 mm was obtained using a pinhole collimator consisting of 0.5 and 0.7 mm diameters. The detector plane was placed at 500 mm from the specimen to record one half of the symmetric scattering pattern, over 180°, on one side of the equatorial plane. Under these conditions, the relative angular resolution of the camera was $\Delta k/k_{min}=0.15$. The performance of the detector and the diffraction apparatus was checked by a standard lupolen specimen (amorphous platelet of polyethylene with 35% crystallinity). It was placed in the beam with its preferential crystalline direction as near the equatorial direction as possible [11].

Octopus vulgaris native hemocyanin was purified and converted to the half-met form by nitrite treatment from the deoxy-protein. Oxy-hemocyanin and half-met hemocyanin were lyophilized with sucrose 1:2 w/w, and the (dry) powders thus obtained were used for small-angle scattering.

3. Results and discussion

Fig. 1 shows small-angle X-ray scattering patterns of lyophilized (a) native hemocyanin and (b) half-met hemocyanin. They show an asymmetric distribution of intensity on the right-hand side of the meridian. The anisotropy of the scattered intensity due to the presence of a preferential direction is evident (the red colour indicates the maximum intensity distribution).

Figs. 2a and 2b show Guinier-plots of the mean scattering curves obtained from the patterns of the native and half-met *Octopus v.* hemocyanins, re-

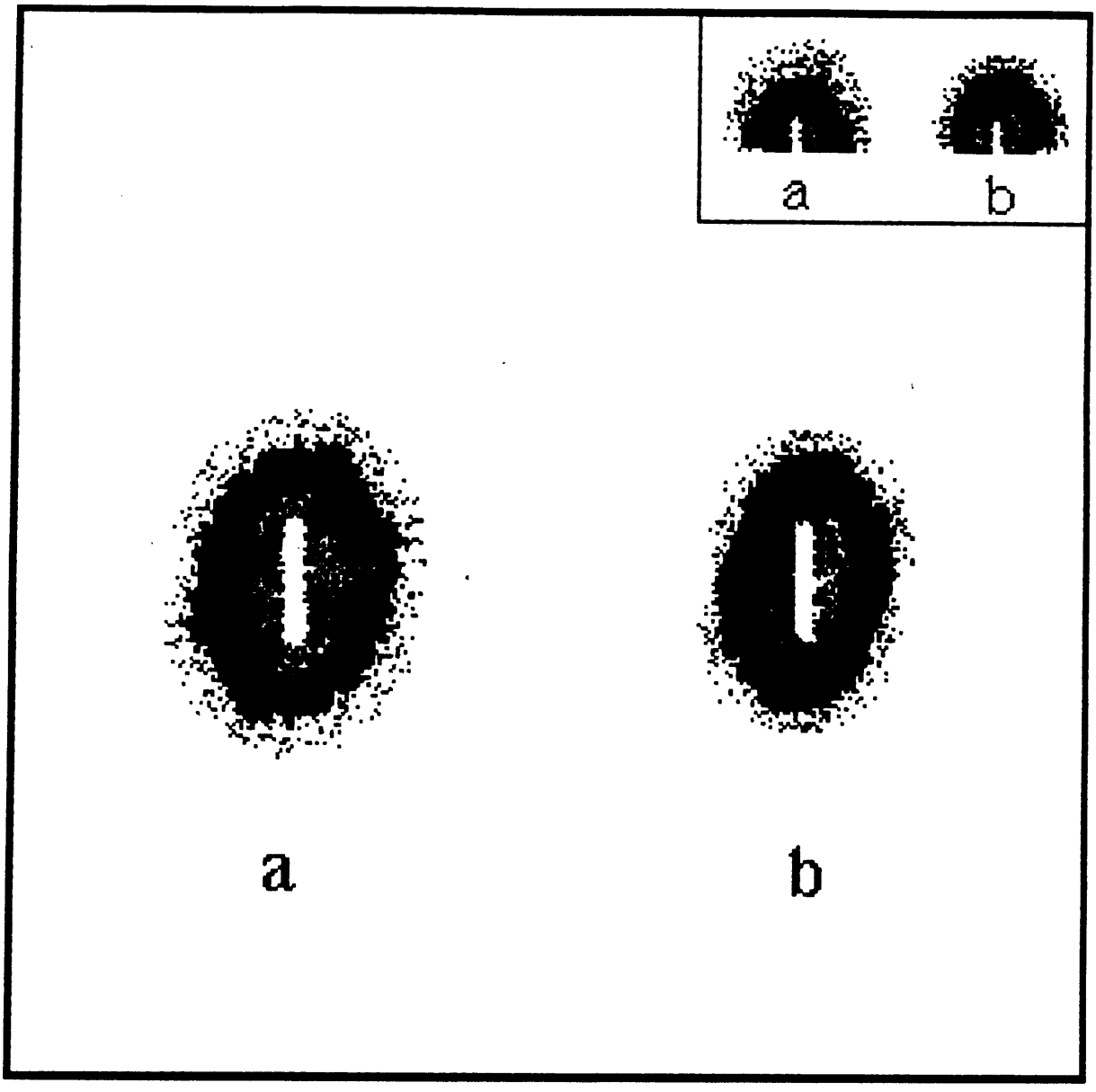


Fig. 1. Small-angle scattering patterns of hemocyanins from *Octopus vulgaris*: (a) native; (b) half-met. The patterns are reconstructed for the whole plane of detection (see text) from the experimental figures in the insert in order to compare with the patterns, usually recorded on film. The red colour indicates the maximum scattering intensity distribution.

spectively. The Guinier-plot of the native hemocyanin is linear, while the Guinier-plot of the half-met hemocyanin shows two different slopes. Thus, there are two distinct scattering units of different dimensions in the half-met hemocyanin. Table 1 lists the average values, within the error range, of the relevant parameters of the native and half-met hemocyanin calculated from the Guinier-plots and the relative models.

Our results for the 49S *Octopus v.* hemocyanin

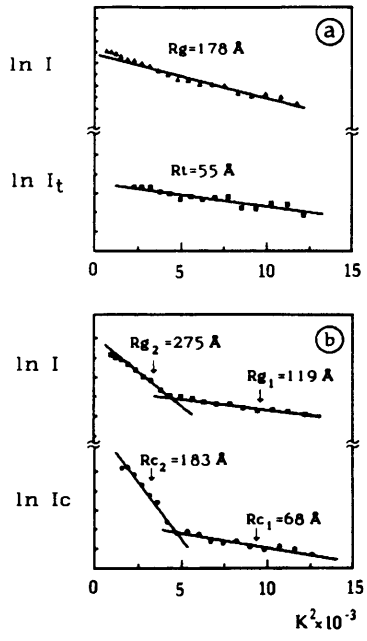


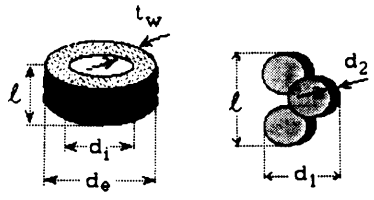
Fig. 2. Guinier-plot of mean scattering curves obtained from experimental diffraction patterns: (a) average radius of gyration (R_g) and radius of gyration corresponding to the thickness (R_t) of the native hemocyanin; (b) average radii of gyration (R_{g1} and R_{g2}) and radii of gyration of the cross-section (R_{c1} and R_{c2}) of half-met hemocyanin.

confirm the expected hollow-cylinder molecular model. But the dimensions of this hollow cylinder, i.e. internal diameter 246 Å, external diameter 408 Å and height 191 Å, as derived from small-angle X-ray scattering, are a little larger than those obtained by electron microscopy [1], namely, internal and external diameters of 170 and 350 Å, respectively, and height of 170 Å [3]. This is because the specimens prepared for electron microscopy suffer shrinkage. The molecular weight determined from the scattered intensity at zero angle [12] is 2.96×10^6 D and compares well with that (2.70×10^6 D) calculated from sedimentation coefficient determination [1].

The measurements on the half-met hemocyanin clearly show two sets of radii of gyration: the smaller radius of gyration corresponds to a smaller-primary-scattering unit, whose dimensions are very different from those of the native hemocyanin (49S) and from those of the 11S subunit [2]; the greater radius of gyration corresponds to a unit which has a larger volume than in the 49S native hemocyanin. This fact can be explained if one assumes that the native molecules undergo dissociation fragmenting into smaller subunits and reassociation into units of different dimensions. It is interesting to note that the d_1 and d_2 dimensions of the smaller unit of half-met hemocyanin form obtained from our experimental data correspond to the double average width and the thickness of the 11S subunit, respectively: in fact, the 11S subunit can be approximated as a circular plate of diameter 130 Å and thickness 60 Å, according to Preux and Gielson [2]. Furthermore, the average length (l) of this primary scattering unit in half-met hemocyanin derivative also corresponds to three times the diameter of the 11S subunit. Therefore, this primary scattering unit can be identified as the fraction 19S, arranged as a trimer of 11S [1], according to the model shown in table 1. The bonding angles

Table 1
Anisometric parameters of the protein hemocyanin from *Octopus vulgaris* obtained by the Adone SAXS camera. R_g: gyration radius of the cross-section; R_t: gyration radius of the thickness; V_i: invariant volume; l: length; d_i, d_e: internal and external diameters; t_w: thickness of the wall: d_1, d_2, diameters of the cross-section of the subunit; a, b: dimensions of the cross-section of the cluster; M_W: molecular weight

Native Hc 49S	Half-met Hc	
Molecule	(1) Sub-unit	(2) Cluster
$R_g = 178$ Å	$R_{g1} = 119$ Å	$R_{g2} = 275$ Å
$R_c = 169$ Å	$R_{c1} = 68$ Å	$R_{c2} = 183$ Å
$R_t = 55$ Å		
$V_i = (252\ Å)^3$	$V_{i1} = (162\ Å)^3$	$V_{i2} = (519\ Å)^3$
model:	model:	model:
hollow cylinder	rod-like shape	rod-like shape
$l = 191$ Å	$l = 388$ Å	$l = 711$ Å
$d_i = 246$ Å	$d_1 = 264$ Å	$a = 626$ Å
$d_e = 408$ Å	$d_2 = 60$ Å	$b = 314$ Å
$t_w = 81$ Å		
$M_W = 2.96 \times 10^6$ D		



between the 11S subunits suggested in this model could be very similar to the original bonding angle found in the 49S molecule which has a fivefold symmetry along its cylindrical axis. The molecular weight of this fraction cannot be estimated with accuracy from the intensity at zero angle because of the presence of the superparticles (clusters), which mask the true contribution of the lower molecular-weight fraction. Also, the exact concentrations of the smaller scattering units (and those of the clusters) are not known in the half-met specimen. We have not been able to observe the presence of the 11S subunit. Further investigations using homogeneous preparation of hemocyanin components common to the different morphological classes are in progress to see whether 11S minimal subunit does exist as an independent scattering unit.

The presence of larger scattering units in half-met hemocyanin could be due to formation of superparticles. The ratio of the volumes between the larger- and smaller-fragment scattering units suggests that the superparticle probably contains thirty 19S fractions (corresponding to, roughly, nine native 49S molecules) interpreted as thirty 11S trimers according to the above-described model. The anisotropy of the scattered intensity, shown in fig. 1, indicates that there may be enhanced preferential scattering in the vicinity of the equatorial direction. This may be explained if one assumes that the aggregates are some form of crystallites or quasi-crystallites formed when the specimens were subject to lower temperatures during the process of lyophilization.

This study has shown that accurate structural data on the morphology and on the relationships of the subunit-to-whole molecule of very high molecular weight can be derived. The technique of the small-angle scattering apparatus with drift-chamber area detector could be used instead of the other techniques such as electron microscopy in order to obtain detailed reconstructions of the molecular architectures.

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