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Abstract

Some preliminary SAXS measurements have been carried out on four different extracellular invertebrate dioxygen carriers. The validity of the procedure has been tested on arthropod hemocyanin: the radii of gyration deduced from SAXS spectra agree with those calculated from the dimensions of the structure determined by X-ray crystallography. The radii of gyration of *Spirographis spallanzanii* chlorocruorin agrees with values deduced from 3D structures obtained with Electron Microscopy. The radius of gyration and the radius of gyration of the cross section of *Octopus vulgaris* hemocyanin are compatible with a ring structure, as deduced from investigations made by Electron Microscopy.

1. Introduction

The tools which can be used in the definition of the quaternary structure of oligomeric proteins are mainly X-ray scattering methods and Transmission Electron Microscopy with computerized 3D reconstruction techniques based on the projection theorem of Fourier transform.

In the case of difficult crystallization, Conventional Transmission Electron Microscopy with Negative Stain (CTEM) can be used with single particles methods, which allow a resolution of 20÷30 Å, so that small species as well as subunits are not suited for this technique; furthermore CTEM gives an adequate picture of the internal organization of molecular aggregates only for particles of high surface/volume ratio while, in the other cases, essentially a surface description, with poor information concerning internal mass distribution is obtained. Finally, flattening and other distortion effects during the sample preparation and the observation may affect the quality of the results.

In this work we have addressed the problem of the quaternary organization of invertebrate dioxygen carriers, hemocyanins and chlorocruorin by a combination of Synchrotron Radiation Small Angle X-ray Scattering (SAXS) and CTEM. Hemocyanins from *Carcinus maenas*, *Cancer pagurus* (Arthopoda, crustacea), *Octopus vulgaris* (Mollusca, octopoda) and the chlorocruorin from *Spirographis spallanzanii* (Annelida, policheta) have been preliminarily studied. All these proteins in the native state are extracellular oligomeric species, whose molecular weight ranges between 4.5×10^5 and 3×10^6 Da, and can be dissociated at relatively high pH (9-9.5) in the absence of divalent cations to yield subunits.

The aim of our research is to describe with SAXS the dissociation products of such proteins as well as the correlation between subunits and the whole molecules.

2. Material and Methods

Hemocyanins were isolated according to [1]. *Spirographis s.* chlorocruorin was extracted and purified as described by [2].

The proteins, stored at -20° C in Tris-HCl 50 mM $CaCl_2$ 20 mM in 20% sucrose, were thawed and diluted in appropiate buffers just immediatly before the measurements.

Dissociated samples were obtained by dissolving aliquots of protein stock solutions in Tris-propionate

50 mM, isopropanol 0.2 M, EDTA 10 mM pH 9.2 for *Octopus v.* hemocyanin and in Tris/propionate 50mM, isopropanol 0.2M, EDTA 10mM, urea 2M pH 9.2 for *Carcinus m.* and *Cancer p.* hemocyanins. *Spirographis s.* chlorocruorin, was dissociated with Tris-HCl 50 mM pH 9 EDTA 10 mM. The measurements on 24S and 16S hemocyanins of *Carcinus m.* were performed by diluting the protein in Tris/propionate, 50mM isopropanol 0.2M $CaCl_2$ 20mM pH 7.0 or in Tris/propionate 50 mM isopropanol 0.2 M EDTA 10 mM pH 8.

The experiments were performed on the D24 SAXS station of LURE facility (Orsay, France). The sample solutions were irradiated with monocromatic radiation of λ = 1.608 Å ($\Delta\lambda/\lambda=10^{-3}$) with a recording time Δt=400 sec. The scattered intensities were recorded on a position sensitive proportional detector, 1205 mm far from the sample, which allowed an angular resolution $\Delta s=1.192\times10^{-}$Å^{-1} ($s=2\sin(\theta)/\lambda$). To collect the data, the program OTOKA from EMBL (Hamburg and Heidelberg) [3] was used. The scattering data were normalized and subjected to background subtraction. For every protein species several solutions, with concentrations ranging from 2 mg/ml to 13 mg/ml were examined.

X-ray scattering data were analyzed by using the Guinier approximation [4]. From the slope of the Guinier plots, obtained at various protein concentration from the linear portions of the scattering curves, the radii of gyration Rg were determined and extrapolated to zero protein concentration.

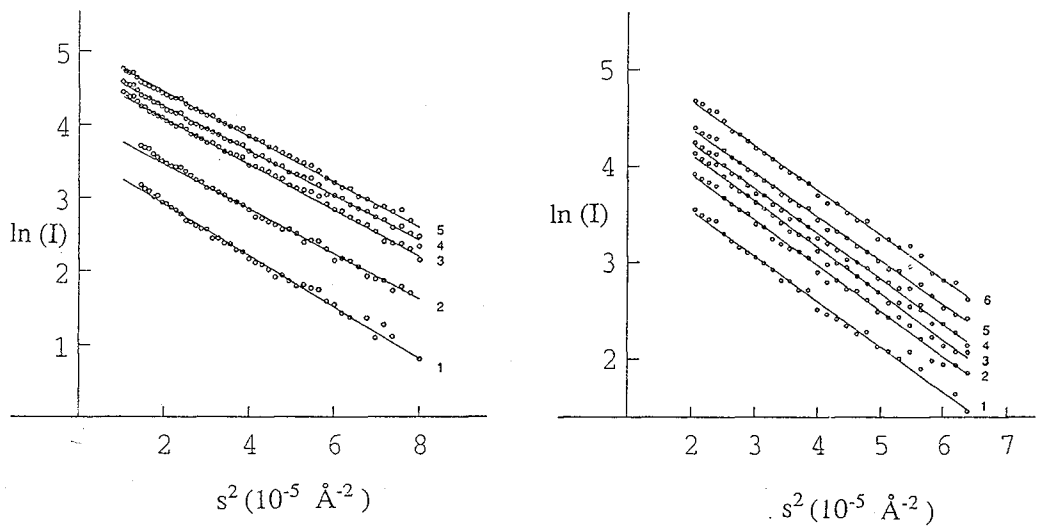

Fig.1 Left: Guinier plots of *Spirographis s.* chlorocruorin (protein concentrations in mg/ml: 1(Curve 1), 3.4(2), 6(3), 8.5(4), 10.2(5)) after buffer subtraction. Right: Guiner plots of *Octopus v.* hemocyanin (protein concentrations 2.8(1), 4.7(2), 6.2(3), 8.75(4), 10.1(5), 14(6)) after buffer subtraction.

3. Results

3.1 *Spirographis spallanzanii* chlorocruorin

A micrograph of this protein shows that it is a two tiered hexagonal disk [6]. It is thought to be made of up to 200 globin chains, organized hierarchically in tetramers, then in main subunits and finally in whole molecules, whose molecular weight is about 3MDa. We examined a solution of the main subunit of *Spirographis s.* chlorocruorin with SAXS, and deduced two radii of gyration: R_{g2}=50 Å corresponds to the main subunit and R_{g1}=23.3 Å, which probably refers to a lower weight dissociation product.

Using a 3D density map of such protein, obtained with CTEM and the Random Conical Tilting procedure [5][6], we calculated the radius of gyration, Rg=46 Å, of the main subunit. This value is slightly lower than that deduced by SAXS probably because of flattening during CTEM preparation and observation [4][5]. From a 3D density map of the whole molecule we also deduced a radius of gyration of

114 Å, which compares with the values 118 Å and 120 Å obtained with SAXS on the phylogenetically related erythrocruorin of *Glossoscolex paulistus* and *Arenicola marina* [8].

3.2 *Octopus vulgaris* hemocyanin

CTEM shows that this molluscan dioxygen carrier is roughly a cylinder, with diameter of 350 Å and height of 170 Å [9]: as commonly found in molluscan hemocyanins it possesses pentameric symmetry. We performed SAXS measurements on the 11S subunit obtained by dissociation [10], which has molecular weight of 250 kDa. Such a subunit shows a radius of gyration Rg=61 Å, and a radius of cross section Rc= 42.7 Å : a structure consisting of a ring with external diameter 90 Å and internal diameter 80 Å is compatible with these dimension. These latter values are subject to errors owing to the use of the invariant Q of the theory [3]: within such errors we can say that there is agreement with the maximum dimension of 113 Å found previously [11].

3.3 Arthropod hemocyanins

X-ray crystallography, CTEM and analytical untracentrifugation have shown that these proteins in 16S aggregation state (MW = 450 kDa) are built up by six equivalent subunits (5S, MW=75kDa) arranged in a trigonal antiprism. With both *Carcinus m.* and *Cancer p.* hemocyanins the 5S subunits exhibit R_g=29 Å. The hexameric 16S species of *Carcinus m.* has R_g=54 Å; at pH around neutrality and in the presence of Ca^{2+}, R_g=65.4 Å is found, attributable to a species resulting from the association of two 16S species (24S, Mw=900kDa), namely the double hexameric form. The values of the radii of gyration are in agreement with the dimensions deduced from X-ray crystallography on *Panulirus interruptus* hemocyanin [12] [13].

Table 1. SAXS parameters of invertebrate dioxygen carriers, extrapolated to infinite dilution.

Protein sample	Rg (Å)	Rc (Å)	Rg (Å) (models)	MW (kDa)
A. marina eritrocruorin (whole protein)	120£			3000£
G. paulistus. eritrocruorin (whole protein)	118£			3200£
S. spallanzanii chlorocruorin (whole protein)			114	3000#
S. spallanzanii chlorocruorin 11S	50 23.3*		46	250#
Octopus vulgaris 11S hemocyanin	61	42.7		250#
Arthropod 5S hemocyanin	29		28$	75#
Arthropod 16S hemocyanin	54		56$	450#
Arthropod 24S hemocyanin	65.4		64$	900#

£Value according to [8]
#Experimental values obtained with techinques different from SAXS
*Radius of gyration of lower molecular weight dissociation products
$Calculations based on [12] and [13]

4. Conclusions and perspectives

In this preliminary work we obtain a survey of some geometrical parameters describing the main subunits of some extracellular dioxygen carriers, summarized in Table 1.

The use of arthropod hemocyanins is a test on the possibilities of SAXS in this field, since the detailed structure of the molecule is known from X-ray crystallography [12].

We found interesting the difference between the values of the radius of gyration of the main subunit of *Spirographis s.* chlorocruorin and *Octopus v.* hemocyanin, two species having approximately the same sedimentation coefficient (11S) and molecular weight (250 kDa). The first is known from CTEM to possess a cavity in the inside [6]. The higher radius of gyration of *Octopus v.* hemocyanin suggests instead a more open structure: a ring, made by five equivalent subunits, covalently linked, having 60 Å diameter each, could agree with previus CTEM studies [14]. Elongated structures or "necklaces" made by 7-8 globular units, corresponding to a CTEM model by [15], give a higher estimated radius of gyration (R_g=78-82 Å). It is worth noting that, on one hand, species with similar sedimentation coefficient and MW value give rather different R_g and that, on the other hand, as in the case of 16S and 24S arthropod hemocyanins, similar R_g values are obtained for species strongly different in MW. These considerations stress the high sensitivity of SAXS approach to geometrical factors.

The solutions examined are remarkably polydisperse. This obliges us to use complex analysis simulations. Improvements on the preparation of the samples have taken place: on this basis we hope to reach a broader description of the quaternary structure of these proteins, beyond the first references just set.

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