Small-Angle X-Ray Scattering Studies of 1/10-Size and 1/20-Size Molecules of *Helix pomatia* α-Hemocyanin

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Hemocyanin, *Helix pomatia*

The small-angle X-ray scattering of 1/10-size molecules (in 0.1 ionic strength Tris buffer pH 8.1) and 1/20-size molecules (in 0.1 ionic strength ethanolamine buffer pH 10.6) of α-hemocyanin of the snail *Helix pomatia* was studied.

The radii of gyration, 8.7 nm and 8.0 nm for 1/10-size and 1/20-size molecules respectively, and the shape of the scattering curves indicate that both have a very anisotropic shape. Comparison of the experimental scattering curve of 1/10-size molecules with theoretical scattering curves gives the best agreement for flat cylinders or quadratic prisms the diameter respectively side of which is about 10 times larger than the height (dimensions side = 21 nm, height 2 nm). The scattering curves of various models composed of larger and smaller subunits were calculated; the best agreement was found also for flat models of similar dimensions.

Introduction

The quarternary structure of α-hemocyanin of *Helix pomatia* has been studied by electron microscopy [1, 2], ultracentrifugation [3, 4], light scattering [5] and small-angle X-ray scattering [6–8]. The model found by the latter method to be equivalent in scattering with the whole molecule was a hollow cylinder with the dimensions: Height 36 nm, inner diameter 15 nm, outer diameter 33 nm [6]; it agrees with the electron microscopic data [1]. From the position of certain minima in the outer part of the small-angle scattering curve it was deduced according to the method of Glatter [9] that the whole molecule consists of a large number of identical (or nearly identical) subunits [7]. The molecule is only stable within a pH-region of 4.5 to 7.3. Outside this pH-region it dissociates into 1/2-size, 1/10-size and 1/20-size molecules. A completely homodisperse solution of the 1/2-size molecules could be prepared and small-angle measurements showed, that the data found for the 1/2-size molecules correspond very well to a hollow cylinder of the same inner and outer diameter but half the height of the whole molecule [8]. This result also agrees with electron microscopic data [1].

Two distinctly different conformations of the 1/10-size molecules have been detected recently by electron microscopy and hydrodynamic measurements [10]. A C(compact)-conformation with well-defined size and shape was observed at high ionic strength, and corresponds to one fifth part of the cylindrical 1/2-size molecule. Upon lowering the ionic strength this conformation loosened to a very flexible molecule, the L(loose)-conformation, which appears as a cluster of 14 or 16 apparently spherical subunits of diameter 5.5 – 6.0 nm. This L-1/10-size molecule dissociates into 1/20-size molecules, by change of pH, which appear as flexible chains of 7 or 8 subunits of similar dimensions. The individual subunits are assumed to correspond to the minimal oxygen-binding units of approximately 50 000 molecular weight [10].

We have calculated a large number of possible models for the 1/10-size molecules composed of larger and smaller spheres and also attempted to correlate the small-angle X-ray scattering of L-1/10-size molecules with the electron microscopy results.

Materials and Methods

Protein solution

α-Hemocyanin of the snail *Helix pomatia* was isolated and stored as described by Heirwegh *et al.* [11] with minor modifications [12, 13]. The preparation of

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solutions of 1/10-size molecules in pH 8.1 Tris buffer ionic strength 0.1, and of 1/20-size molecules in pH 10.6 Ethanolamine buffer ionic strength 0.1, was performed as described by Siezen and van Driel [13]. The protein concentration was determined according to Heirwegh *et al.* [11].

Sedimentation analysis

Sedimentation velocity experiments were carried out in a Beckman Spinco model E analytical ultracentrifuge, equipped with electronic speed control and Schlieren optics, at 20 °C.

Partial specific volume

The apparent partial specific volume $\bar{v}'$ of the proteins in the solutions used was determined by the oscillator method [14, 15] using the digital densitometer device DMA 02/C at 20 °C. The $\bar{v}'$-values found for 1/10-size and 1/20-size molecules were 0.722 cm^3/g and 0.725 cm^3/g, respectively.

Small-angle X-ray scattering

The small-angle measurements were made with a highly stabilized X-ray generator (Philips PW 1140) using a copper tube. For the collimation of the X-ray beam a Kratky camera [16, 17] was used. The hemocyanin solutions were placed in Mark capillaries (diameter about 0.1 cm) and irradiated at a temperature of 20 °C. The scattered intensities were recorded by using a proportional counter with pulse-height discriminator as a detector for the Cu lines Kα and Kβ. Elimination of the Kβ-line was effected by using a computer programme [18].

An electronically programmable step scanning device [19, 20] allowed automatic operation. The scattered intensities were measured in the angle range between 0.002 – 0.1 radians. Each single solution was exposed to X-rays for about 12 hours. During this period its scattering curve was recorded several times, whereby as a rule 10^5 pulses were registered for each of the 72 measuring points. The evaluation of the scattering data was done by using the computer programme of Zipper [21]. The scattering curves of the models were calculated by a computer programme of Glatter *.

The slit collimation of the camera causes the "smearing" of the scattering curves; these collimation effects caused by the line shape of the primary beam were eliminated using the computer programme described by Glatter [22].

Results and Discussion

Sedimentation analysis

Sedimentation analysis (Fig. 1) showed that the pH 8.1 solution contained mainly 1/10-size molecules and small amounts of 1/20-size molecules (4%) and 2/10-size molecules (6%), whereas the pH 10.6 solution contained mainly 1/20-size molecules and larger amounts of 1/10-size (15%) and smaller ones of 2/10-size molecules (5%).

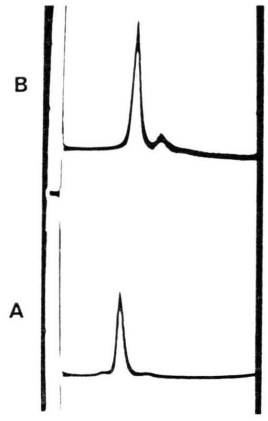

Fig. 1. Sedimentation of *H. pomatia* α-hemocyanin, 20 °C. A. 1/10-size molecules; 5 mg/ml; pH 8.1 Tris buffer, ionic strength 0.1; sedimented for 20 min at 52000 rpm. B. 1/20-size molecules; 6 mg/ml; pH 10.6 Ethanolamine buffer, ionic strength 0.1; sedimented for 42 min at 52000 rpm.

Small-angle scattering

A concentration series of the 1/10-size molecules and the 1/20-size molecules was studied. The protein concentrations of the 1/10-size molecules were 39.9, 26.3, 13.3 and 6.6 mg/ml, those of the 1/20-size molecules 48.3, 18.9, 9.5 and 4.8 mg/ml. The scattering of each solution was measured several times at 20 °C.

From the scattering curves the radius of gyration R and the molecular-weight M [23] were calculated. The equations used for these calculations have been published and can be found in summarizing papers [24–26].

Information on the overall shape of the molecules was obtained by comparing the experimental scattering curves and data with the theoretical curves and data of various models.

The data obtained are afflicted with some uncertainty since the solutions were not completely homodisperse. As far as possible the degree of uncertainty caused by the small amounts of larger and smaller aggregates was calculated. These semi-quantitative data already give some valuable hints at the size and shape of the 1/10-size and 1/20-size molecules.

* Publication in preparation.

Radius of gyration

The radii of gyration were calculated from the slope of the innermost portions of the scattering curves. In Fig. 2 the experimentally obtained, slit-smeared scattering curves at small angles of 1/10-size molecules are shown in Guinier plot. The apparent (slit-smeared) values of the radius of gyration $\tilde{R}$, calculated from the slope of the straight lines, are summarized in the Table and plotted in Fig. 3 *versus* the concentration of the solutions.

Fig. 4 shows the innermost portions of the scattering curves of the 1/20-size molecules in Guinier plot. The concentration of the solutions and the $\tilde{R}$-value found from the slopes of the plots are also summarized in the Table and plotted in Fig. 3.

After desmearing and extrapolation to zero concentration for the 1/10-size molecules a radius of gyration of 8.7 nm was found and for the 1/20-size molecules a value of 8.0 nm. Taking into considera-

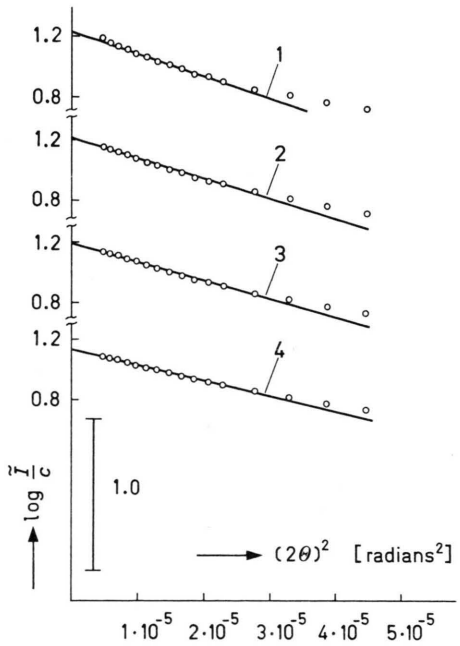

Fig. 2. Innermost portions of the slit-smeared scattering curves of 1/10-size hemocyanin molecuels in solution in Guinier plot. The concentrations c of the solutions (1—4) and the apparent radii of gyration $\tilde{R}$ calculated from the straight-lined part of the plots are summarized in the Table. $\tilde{I}$: Slit smeared scattering intensity; 2θ: Scattering angle.

Table. Apparent radii of gyration $\tilde{R}$ of 1/10- and 1/20-size hemocyanin molecules calculated for solutions of various protein concentrations c. The numbers of solutions correspond to those given in Figs 2 and 4.

	1/10-size			1/20-size	
No.	c [mg/ml]	$\tilde{R}$ [nm]	No.	c [mg/ml]	$\tilde{R}$ [nm]
1	6.6	7.8	1	4.8	6.9
2	13.3	7.4	2	9.5	6.3
3	26.3	7.3	3	18.9	5.9
4	39.9	6.5	4	48.3	4.1

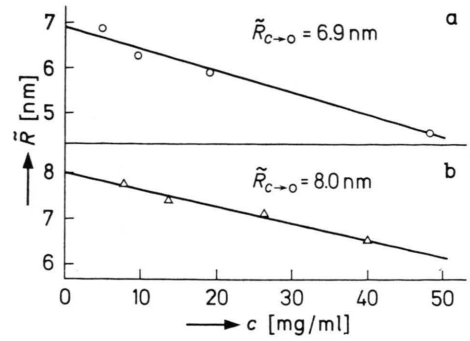

Fig. 3. Apparent radii of gyration $\tilde{R}$ calculated from the curves shown in Fig. 2 for the 1/10-size molecules (lower curve b) and in Fig. 4 for the 1/20-size molecules (upper curve a) *versus* the concentration c of the particles in solution. The $\tilde{R}$-value extrapolated to zero concentrations is given.

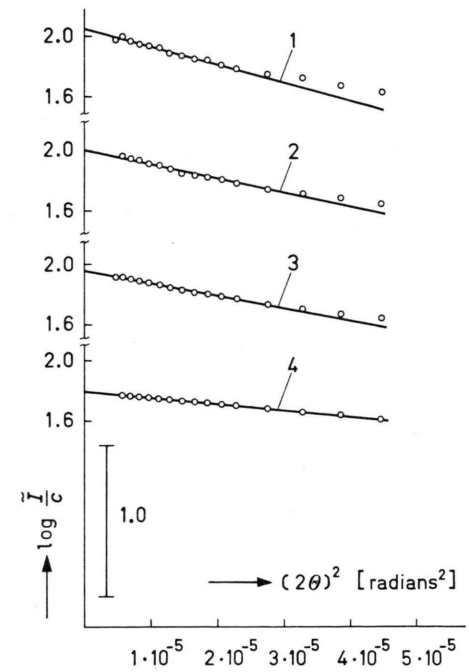

Fig. 4. Innermost portions of the slit-smeared scattering curves of 1/20-size molecules in solution in Guinier plot. The concentrations c of the solutions (1—4) and the apparent radii of gyration $\tilde{R}$ calculated from the straight-lined parts of the plots are given in the Table.

tion the small amounts of other aggregates (see ultracentrifugation) and the fact that the R-value is a Z-average, the following uncertainty should be added to the values found for the 1/10-size and 1/20-size molecules.

1/10 – size molecules: $R = 8.7 \pm 0.3$ nm,
1/20 – size molecules: $R = 8.0 \pm 0.3$ nm.

(With this calculation it was assumed that the radius of gyration of the 2/10-size molecules is not larger than that found for the 1/2-size molecules: $R =$ 14 nm [8].)

Molecular weight

The molecular weight can be determined from the scattered intensity at zero angle and from the intensity of the incident beam [23]. The intensity of the incident beam was measured by using a calibrated Lupolen^R sample [27, 28]. For the calculation of the molecular weight the partial specific volume and the concentration of the dissolved particle have to be determined as accurately as possible. The values obtained are

1/10-size molecules $M = 600\,000 \pm 20\,000$,
1/20-size molecules $M = 330\,000 \pm 40\,000$.

The obtained M-value is a weight-average; the uncertainties caused by the small amounts of other aggregates are indicated.

These values are clearly lower than those obtained by equilibrium sedimentation: 1/10-size molecules: $730\,000 \pm 30\,000$ and 1/20-size molecules: $365\,000 \pm 20\,000$ [10]. No explanation is available for this difference.

Shape

1/10-size molecules

The high value of the radius of gyration obtained for the 1/10-size molecules indicates that they must have a rather anisotropic shape, for the following reason: if we assume that the 1/10-size molecules ($M = 600\,000$) are unhydrated and have a spherical shape the radius of gyration of this sphere would be only 4.3 nm; assuming the same degree of hydration as found for the whole and 1/2-size hemocyanin molecules (0.43 g H_2O per 1 g protein) a radius of gyration of 5 nm is calculated for the spherical particle. Both values are much lower than the experimentally found value of 8.7 nm indicating that the molecules must be rather anisotropic (largest dimension about 20 to 30 nm). The form of the scattering curve also agrees only with anisotropic models such as flat cylinders or prisms and deviates strongly from the curves of isotropic bodies. The best agreement was found for a quadratic prism of an axial ratio 1 : 1 : 0.1 as shown in Fig. 5. The dimensions of the prism which has the same radius of gyration as experimentally found are 21.0 nm × 21.0 nm × 2.1 nm. A flat cylinder of similar dimensions also gives a good agreement.

The same results were obtained by studying the 1/10-size molecules in 0.05 M borate-HCl buffer, pH 8.2 [6] and 0.1 M phosphate buffer pH 7.0 by treatment with diethylpyrocarbonate 5% (v/v in methanol) per mg protein [8].

Furthermore a large number of models composed of larger and smaller spherical subunits were calculated; the overall shapes of the models are similar to those seen on electron microscopic pictures recently [10]. All isotropic models could be excluded for the reasons given above. In Fig. 6 some models are

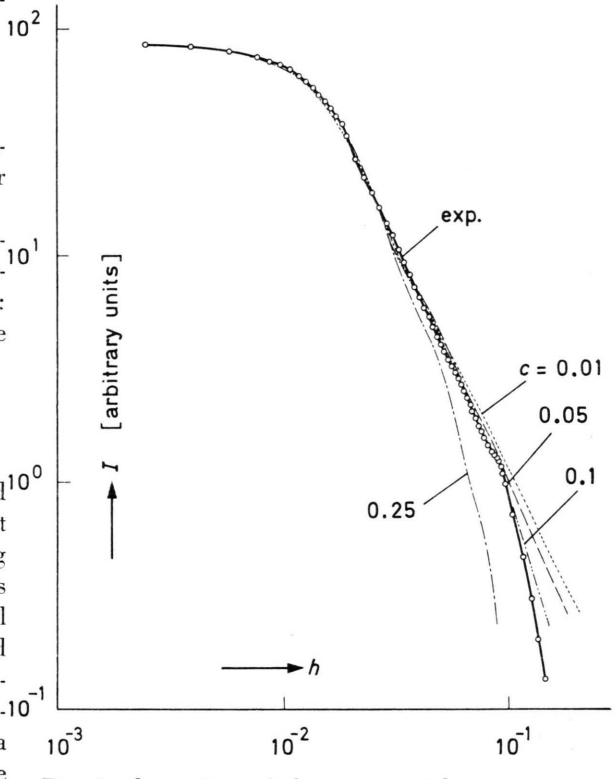

Fig. 5. Comparison of the experimental scattering curve (–○–○–) of the 1/10-size molecules with theoretical scattering curves of flat prisms which have axial ratios 1:1:c; c has the value 0.01, 0.05, 0.10 and 0.25 as indicated. $h = (2\pi/\lambda)\sin(2\theta)$, whereby 2θ is the scattering angle and λ the wavelength of the CuKα-line used (0.154 nm).

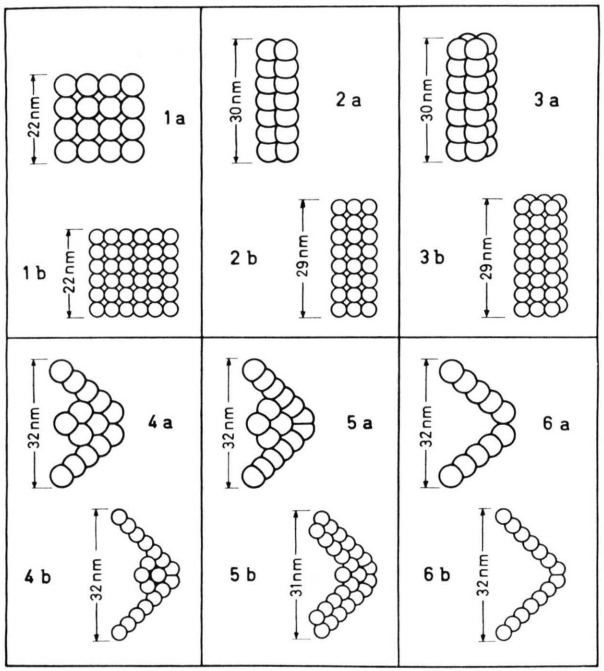

Fig. 6. Various models for the 1/10-size hemocyanin molecules similar to the shapes seen on electron microscopic pictures [10]. The models are composed of spherical subunits whereby the diameter of the larger spheres is 6 nm, that of the smaller ones 4 nm. Models 1—5 (a and b) have the same radius of gyration ($R=8.1$ nm) as found experimentally for the 1/10-size molecules. The radius of gyration of the models 6 a and 6 b is larger ($R=9.8$ nm); for the comparison of the scattering curves of these models with the experimental curve (Fig. 7) the abscissa scale therefore was normalized to $h\,R$, that is, all scattering curves compared belong to particles of the same R-value.

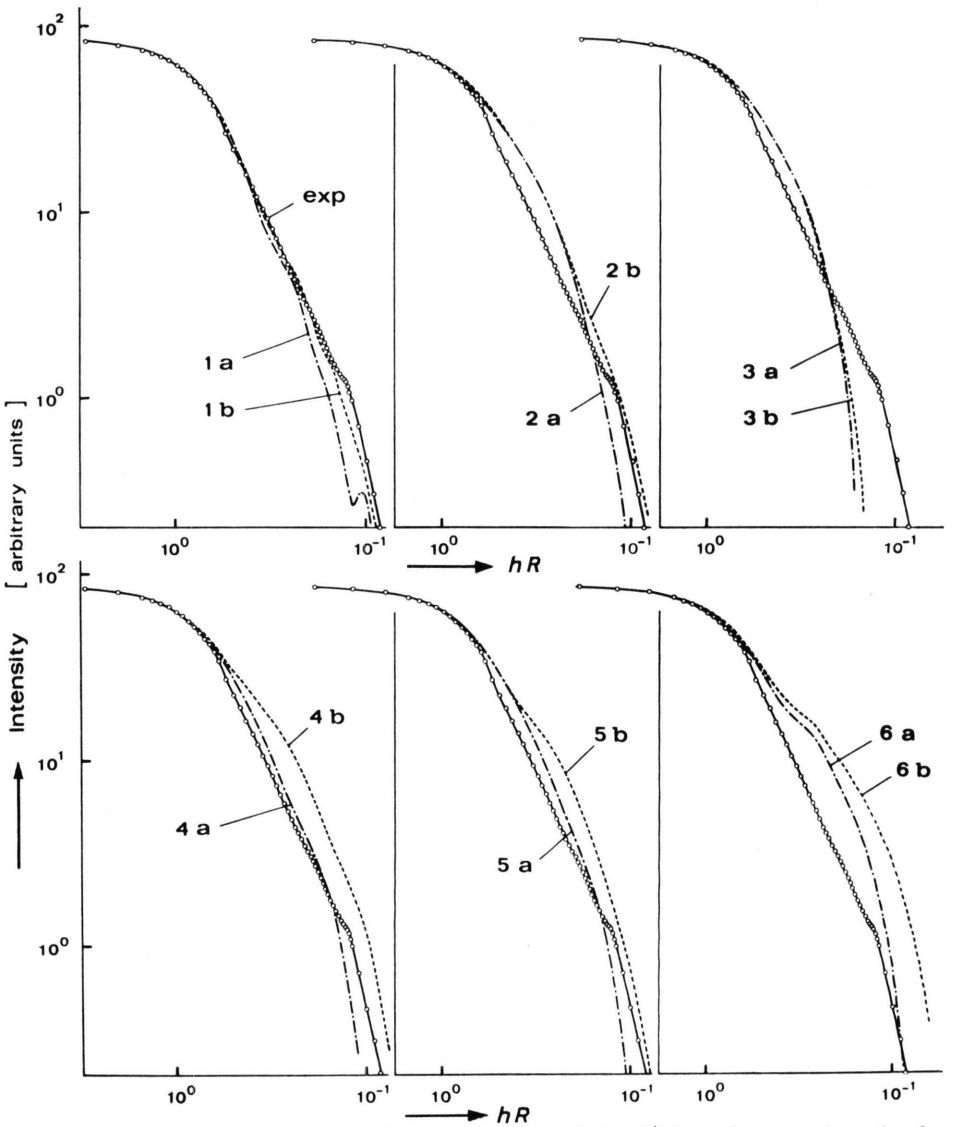

Fig. 7. Comparison of the experimental scattering curve of the 1/10-size hemocyanin molecules (circles) with the models shown in Fig. 6. The intensity is given in arbitrary units, the abscissa scale in $h\,R$ (compare legend of Fig. 6); log-log plot is used. $h=(2\,\pi/\lambda)\sin(2\,\theta)$ and R is the radius of gyration.

shown composed of larger spheres (diameter 6 nm) [10] or smaller spheres (diameter 4 nm) [7]. The models 1 a to 5 a and 1 b to 5 b have the same radius of gyration as experimentally found for the tenths $R = 8.7$ nm. In Fig. 7 the scattering curves of the models are compared with the experimental scattering curve of the 1/10-size molecules. Flat models such as shown in Fig. 6 (model 1 a or 1 b) show the largest similarity; 4 a also shows a good agreement. Models which are more elongated than flat (6 a and 6 b) can be excluded. For all models similar in scattering the largest dimension is between 22 and 32 nm.

Since the sample was not completely homodisperse only a qualitative comparison of the curves was possible, but the result that flat anisotropic models fit the scattering curve best is in agreement with the shape of the 1/10-size molecules as determined by electron microscopy [10].

1/20-size molecules

The scattering curve of 1/20-size molecules indicates that they are also rather anisotropic. The high value of the radius of gyration confirms this result: A sphere of $M = 330\,000$ would have an R-value of 3.5 nm assuming no hydration and a value of 4.1 nm assuming a hydration of 0.43 g H_2O per 1 g protein. Both values are much lower than the experimentally found R-value of 8.0 nm. This high value indicates that the largest dimensions of the 1/20-size molecules cannot be much smaller than those of the 1/10-size molecules (20 – 30 nm); this result is an agreement with electron microscopic pictures which show also rather elongated molecules [10].

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