Solution Properties of Phycocyanin. II. Studies of the Molecular Shape and Size by Using the Shell Model**

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The number-average molecular weight and intrinsic viscosity of phycocyanin, isolated from *Porphyra tenera*, have been determined by osmotic pressure and viscosity measurements at various values of pH and ionic strength. The molecular shape and size for phycocyanin aggregates were also discussed by comparing the experimental values of sedimentation coefficient and intrinsic viscosity with the calculated results obtained by using the shell model theory. It is shown that the aggregate of phycocyanin at pH 5.4 is a hexamer of molecular weight 256000 and the structure consists of six juxtaposed monomers. The monomer is a prolate ellipsoid of 35 Å in diameter and 105 Å length. This structure for the hexamer is consistent with the model proposed by Jennings. At pH 6.8, it is shown that the number-average molecular weight of phycocyanin depends on the ionic strength in solution.

In a previous paper,[1] we studied the dissociation-association equilibrium of phycocyanin, isolated from red algae, *Porphyra tenera*, by sedimentation transportation measurements at pH 6.8 and 5.4 and ionic strengths of 0.1 and 0.2. The sedimentation velocity data indicated that the predominant phycocyanin aggregate was a trimer at pH 6.8 and a hexamer at pH 5.4. Moreover, the dissociation-association system of phycocyanin was presumed to be trimer⇌monomer at pH 6.8 and hexamer⇌monomer at pH 5.4.

Berns and Edwards[2] observed phycocyanin aggregates with an electron microscope. They suggested that the higher aggregate of phycocyanin consisted of six globular particles, and, also, that the shape of the hexagonal aggregate was that of an oblate or prolate ellipsoid with an axial ratio of approximately 4 : 1. Scott and Berns[3] performed diffusion and viscometric measurements on phycocyanin solutions, and proposed a round structure with a central hole for the phycocyanin aggregate. Jennings[4] discussed the size and shape of the phycocyanin aggregate on basis of an amino acid composition analysis reported by Berns *et al.*[5] and proposed that the hexamer was an aggregate of six monomers, in juxtaposition, with their major axes parallel so as to form a ring structure, as shown in Fig. 1. Although some investigators proposed a trimer for the lower aggregate of phycocyanin, the shape and size of this aggregate have not been discussed.

Bloomfield *et al.*[6,7] reported a method of estimating the size and conformation of macromolecules in terms of the number of equivalent spherical subunits. In this procedure, the complex structures are replaced by a surface shell consisting of many small spheres, and hence this approximation for a macromolecular complex structure is called the "shell model." Bloomfield *et al.*[7] studied the size of phycocyanin using their theory and proposed that the phycocyanin monomer was a single sphere having a 26 Å radius or a linear array of three spheres, each having an 18 Å radius.

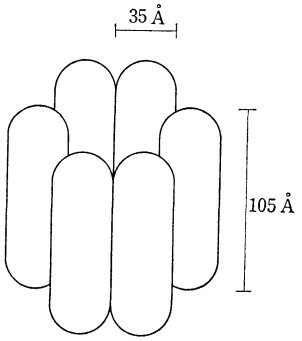

Fig. 1. Schematic model of the phycocyanin hexamer proposed by Jennings[4].

Tsuda[8,9] presented an expression for the intrinsic viscosity of macromolecules based on Kirkwood's approximation for the frictional coefficient. It was found that the experimental results obtained from the measurements of sedimentation coefficient and intrinsic viscosity for many biological macromolecules, such as fibrinogen, hemoglobin and hemocyanin, can be reasonably explained by the shell model.[7,8]

In this paper, the molecular weight and the intrinsic viscosity were measured in phycocyanin solutions under several sets of conditions, that is, pH 6.8 and 5.4, and ionic strength (μ) 0.01, 0.1 and 0.2. The molecular size and shape of this protein were discussed by using the theory of the frictional coefficient of the multi-subunit structure reported by Bloomfield *et al.*[7] and Tsuda.[8,9]

Experimental and Calculation

Treatment of Phycocyanin. The phycocyanin used in this study was obtained from dried *Porphyra tenera* by repeated precipitation with ammonium sulfate, and the process for sample preparation and the concentration determination of the protein solution were described in detail in part I.[1].

Osmotic Pressure Measurements. Osmotic pressure measurements of phycocyanin were conducted in buffer solution at 25.0 °C with a Model 502 High Speed Membrane Osmometer. Phosphate (pH 6.8, μ 0.01, 0.1 and 0.2) and acetate (pH 5.4, μ 0.1 and 0.2) buffer solutions were used in these determinations. The membrane was Gel-cellophane #600 supplied by Tokyo Cellophane, and was conditioned by the

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"subsequent transfer method" from water to buffer solution. Prior to use, the membrane was equilibrated overnight in the appropriate buffer solutions. The number-average molecular weight and the second virial coefficient were obtained by the usual method.

Viscosity Measurements. Viscosity measurements were made in the buffer solution at 25.0 °C, under the following conditions: pH 6.8 and 5.4; μ 0.1 and 0.2. An Ubbelohde dilution type viscometer was used for the measurements. Kinetic energy corrections were not made. The intrinsic viscosity was obtained by using Huggins' equation.

Results and Discussion

The osmotic pressure data are shown in Fig. 2, plotted as π/c *versus* c. As shown in this figure, the concentration dependence of π/c was small for all systems. Therefore, the second virial coefficients of all systems were considered to be nearly zero. The values of π/c at pH 5.4 show the same value irrespective of the ionic strength (0.1 or 0.2), while the π/c values at pH 6.8 increased with decreasing ionic strength of the solution.

The number-average molecular weight at pH 5.4 was 256000. At pH 6.8, the number-average molecular weights were 228000 for μ 0.2, 172000 for μ 0.1 and 154000 for μ 0.01.

We consider that the change in the molecular weight of phycocyanin with the variation of ionic strength at pH 6.8 arises from the difference in the dissociation-association reaction of the protein. In the previous paper, it was shown that the sedimentation coefficient of phycocyanin did not change markedly with changing ionic strength.[1] The results seem to be a consequence of measuring the sedimentation coefficient at too low concentration. As pointed out previously,[1] the dissociation-association equilibrium for phycocyanin at low concentration was trimer$\rightleftarrows$monomer, and the predominant species was a trimer. However, the results from the present study suggest that various aggregates, having different molecular weights, coexist in higher concentration regions. At pH 5.4, the

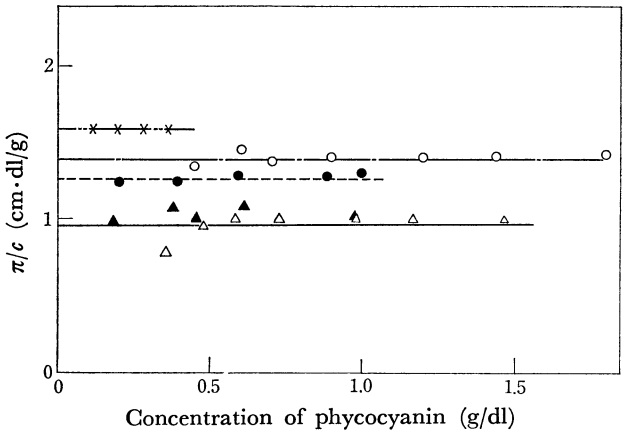

Fig. 2. Plots of π/c *versus* phycocyanin concentration in five different buffer solutions at 25.0 °C. ○, phosphate buffer, pH 6.8 and μ=0.1; ●, phosphate buffer, pH 6.8 and μ=0.2; ×, phosphate buffer, pH 6.8 and μ=0.01; △, acetate buffer, pH 5.4 and μ=0.1; ▲, acetate buffer, pH 5.4 and μ=0.2.

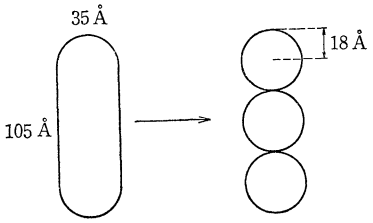

Fig. 3. Monomer model proposed by Jennings[4] treatment according to the shell model.

phycocyanin molecule seems to be relatively stable in solution.

Figure 3 shows the model of the phycocyanin monomer for the calculation of theoretical values of sedimentation coefficient and intrinsic viscosity. This model is the same one as that proposed by Jennings[4]. The monomer structure was assumed to be an array of three identical spheres of radius of 18 Å as shown in Fig. 3. Also the molecular weight of the monomer was estimated to be 42000. This value was about 1/6 of 256000, which was the molecular weight of hexamer obtained from an osmotic pressure measurements.

Viscosity data for phycocyanin in various buffer solutions at 25.0 °C are shown in Fig. 4. All plots of η_{sp}/c against c under various conditions (pH 6.8, μ 0.2, and pH 5.4, μ 0.1 and 0.2) displayed an increase with decrease in concentration in lower concentration regions. This viscosity behavior is generally found for polyelectrolyte solutions. The viscosity characteristic for phycocyanin may be attributed to the charge effects on the protein molecule. Intrinsic viscosities of phycocyanin reported in this paper were determined by linear extrapolation of plots of η_{sp}/c against c in a higher concentration range to infinite dilution. From this extrapolation the values of $[\eta]$ for phycocyanin aggregates were found to be 3.92 ml/g at pH 5.4 and 4.32 ml/g at pH 6.8.

According to the shell model theory, the translational frictional coefficient, f, of a molecule composed of n identical spherical subunits, may be expressed by following equation[8] which is derived from the Kirkwood theory for irreversible processes in solutions of macromolecules.[10]

$$f = \frac{6\pi\eta_0 n^2 r}{n + r\sum_i \sum_{j(\neq i)} R_{ij}^{-1}} \tag{1}$$

where r is the radius of the sphere, η_0 the solvent viscosity and R_{ij} the distance between the i-th and j-th subunit.

In a fluid medium, the rate of sedimentation for solute depends on the translational frictional coefficient, f. Therefore, the sedimentation constant, s_0, is a function of the frictional coefficient and may be expressed by Eq. (2):

$$s_0 = \frac{M(1-\bar{v}\rho)}{fN_A} \tag{2}$$

where M is the molecular weight, $\bar{v}$ the partial specific volume of solute, ρ the density of solvent and N_A Avogadro's number.

On the other hand, the intrinsic viscosity of a macro-

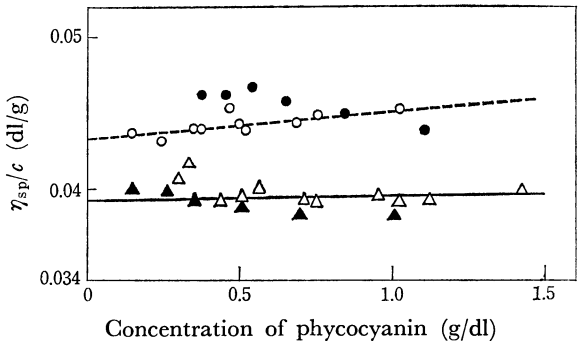

Fig. 4. Plots of η_{sp}/c *versus* concentration of phycocyanin in four different buffer solutions at 25.0 °C. ○, phosphate buffer, pH 6.8 and $\mu=0.1$; ●, phosphate buffer, pH 6.8 and $\mu=0.2$; △, acetate buffer, pH 5.4 and $\mu=0.1$; ▲, acetate buffer, pH 5.4 and $\mu=0.2$.

molecule is related to the rotational frictional property. A general expression for intrinsic viscosity of a complex structure composed of different subunits is

$$[\eta] = \left(\frac{4\pi}{3}\right)\left(\frac{N_A}{M}\right)B \tag{3}$$

where B is a parameter related to the molecular shape and size of a complex macromolecule and its form is given by Eqs. (29)—(33) of Ref. 8.

The intrinsic viscosity and sedimentation constant for various model structures of phycocyanin aggregate were calculated by a computer using Eqs (1), (2) and (3). In these calculations, the monomer structure was assumed to be an array of three identical spheres with a radius of 18 Å, and the monomer molecular weight of 42000, estimated from the osmotic pressure measurements, was used.

For the monomer model the intrinsic viscosity was calculated to be 4.2 ml/g, and the sedimentation constant to be 3.5×10^{-13} s, respectively. The s_0-value of 3.5×10^{-13} s agrees with the experimental value (3.2×10^{-13} s) obtained by Hattori *et al.*[11] Therefore, the shape of the phycocyanin monomer may be a prolate spheroid with an axial ratio of approximately 3 : 1. Since the experimental value of $[\eta]$ for the monomer has not yet been reported, the calculated value of the intrinsic viscosity cannot be compared with the experimental value.

The values of $[\eta]$ and of s_0 for Jennings' model and other assumed hexameric structure of phycocyanin were calculated by the shell theory. An $[\eta]$ value of 4.08 ml/g and an s_0-value of 11.8×10^{-13} s were calculated for Jennings' model. These values are in reasonable agreement with the exprimental values ($[\eta]=$ 3.92 ml/g and $s_0=10.8\times10^{-13}$ s[1]) obtained at pH 5.4. Therefore, the aggregate of the protein seems to be a hexamer at pH 5.4. The hexamer of phycocyanin can be considered to be an aggregate of six prolate monomers. The prolate monomers are associated side by side with their major axes parallel to the hollow core of the hexamer. Such a structure for hexamer would have an axial ratio close to unity.

From the results of osmotic pressure measurements at pH 6.8, it might be supposed that the aggregate at this pH value is a tetramer. However, the results of the calculations of $[\eta]$ and s_0 for some tetrameric models of phycocyanin aggregate eliminated this possibility.

TABLE 1. CALCULATED VALUES OF INTRINSIC VISCOSITY AND SEDIMENTATION COEFFICIENT FOR THE ASSUMED MODEL OF THE PHYCOCYANIN TRIMER

At pH 6.8 the experimental value of $[\eta]$ is 3.92 ml/g and s_0 is 6.4s.

Model	$[\eta]$ (ml/g)	s_0 (S)
Fig. 5-a	4.47	7.16
Fig. 5-b	3.65	7.74

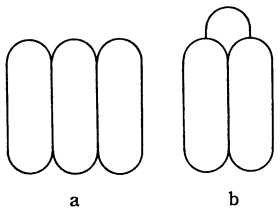

Fig. 5. Schematic models of phycocyanin trimer.

Table 1 shows the results of the calculations for some trimer models depicted in Figs. 5-a and 5-b. As is evident from Table 1, the calculated value of $[\eta]$ for the assumed structure of trimer, shows a remarkable deviation from the experimental value although it satisfactorily explains the sedimentation behavior.

From these results, it seems to be impossible to represent the trimer by a self-consistent model for both the sedimentation constant and the intrinsic viscosity. However, it should be remembered that the sedimentation constant measurements were carried out at concentrations below 0.5 g/dl. On the other hand, both the intrinsic viscosity and the osmotic pressure were measured at higher concentration. The existence of higher order aggregate cannot be neglected in the higher concentration regions at pH 6.8. It seems that the values of intrinsic viscosity and number-average molecular weight obtained in this study are averages for a mixture of trimer and higher order aggregates (probably hexamers).

The weight fraction for each species in the mixture can be calculated from the number-average molecular weight, assuming a hexamer⇄trimer equilibrium for the mixture. The results indicate the weight fraction of trimer is 0.46 and of hexamer, 0.54. By considering the intrinsic viscosity of the mixture as the weight average value, the value of $[\eta]$ for the mixture was calculated from the intrinsic viscosity estimated for trimer and hexamer. The calculated value (4.26 ml/g) of $[\eta]$ for the mixture agrees well with the experimental value (4.32 ml/g). Agreement between calculated and exprimental values suggests that the coexistence of higher order aggregates in higher concentration regions cannot be neglected.

Scheraga and Mandelkern[12] derived the following equation for a revolutional ellipsoid:

$$s_0[\eta]^{1/3} = \frac{M^{2/3}(1-\bar{v}\rho)\beta}{N_A\eta_0} \tag{4}$$

where η_0 is the viscosity of the solvent. They indicated that the quantity β is a function of the axial ratio of the ellipsoid, and has a value of $2.12—2.20 \times 10^6$ for the prolate ellipsoild when the axial ratio is less than 5.

The β values for the various aggregates of phycocyanin were calculated using Eq. (4). In the calculation of β for the monomer, an intrinsic viscosity value of 4.20 ml/g was used, which was obtained by computation with the shell model as shown in Fig. 3. The β values for the monomer, the trimer and the hexamer of phycocyanin are 2.00×10^6, 1.95×10^6 and 2.05×10^6, respectively. The results agree with the theoretical value for a prolate ellipsoid having an axial ratio lower than 5.

From the β-values obtained, the geometrical structures for phycocyanin aggregates used in this study seem to be consistent; that is, the monomer model is that as shown in Fig. 3, the trimer model that is Fig. 5-a or 5-b, and the hexamer model that in Fig. 1.

Conclusion

We conclude that the aggregate of phycocyanin at pH 5.4 is a hexamer. From an analysis of the sedimentation constant and intrinsic viscosity by the shell model, we proposed that the hexamer structurally consists of an aggregate of six juxtaposed monomers, whose major axes are parallel to each other forming a closed ring structure. Each monomer is represented by a prolate ellipsoid with axial lengths of 35 Å and 105 Å. This structure for the phycocyanin hexamer is identical to that proposed by Jennings.

The phycocyanin aggregates at pH 6.8 in higher concentration regions are considered to be composed of various aggregates with different structures. Therefore, a definite structural description of the phycocyanin aggregates at pH 6.8 cannot be given.

It has shown from the values of β that the aggregates of phycocyanin, that is, monomer, trimer and hexamer, are prolate ellipsoids with an axial ratio less than 5.

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