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Solution Properties of Phycocyanin. VI. The Characterization of the Phycocyanin Monomer†

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The monomer molecular weight of phycocyanin was determined by sedimentation equilibrium measurements in 6 mol/l guanidine hydrochloride (GndCl) and by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. The former measurement gave the monomer molecular weight of 4.0×10^4. From the latter, the phycocyanin was found to be composed of two kinds of subunits, the molecular weights of which were 1.7×10^4 and 2.0×10^4. Assumimg that the phycocyanin monomer consists of two polypeptide chains, the molecular weight becomes 3.7×10^4. These values coincide with the value of 4.2×10^4 assumed in our previous works, which corresponds to one-sixth of the hexamer molecular weight. The sedimentation coefficient, $s_{25\mathrm{w}}$, was 2.21 Svedberg unit, and the intrinsic viscosity in 6 mol/l GndCl was 19.5 ml/g, at 25.0 °C. From these values, it was clear that the phycocyanin monomer was unfolded in the solvent.

Phycocyanin, isolated from red- or blue-green algae, undergoes reversible self-associating reactions according to changes in the environment, such as in the pH, the ionic strength, the temperature, and the protein concentration of the solution.

In our previous papers,[1,2] we postulated, on the basis of the results of the sedimentation equilibrium measurements at various temperatures, that the dissociation-association behavior of phycocyanin could be explained by assuming a monomer$\rightleftharpoons$trimer$\rightleftharpoons$tetramer equilibrium system at pH 6.8 and the ionic strength (I) of 0.1. On the other hand, the dissociation-association of the same sample at pH 5.4 and I 0.1 was determined to follow a monomer$\rightleftharpoons$hexamer equilibrium system. The approach used in the estimation of the equilibrium constant from the centrifugal data requires the value of the monomer molecular weight, M_1. The molecular weight of the phycocyanin hexamer has been obtained as 2.56×10^5 from osmotic measurements at pH 5.4 and I 0.1.[3] However, the value of M_1 could not be determined in aqueous solutions because of the dissociation behavior in a lower-concentration region and because of the association behavior in a higher-concentration region. Thus, the value of 4.2×10^4 was assumed for M_1 in the previous works; this value corresponds to one-sixth of the value of 2.56×10^5 for hexamer.

This work was undertaken in order to decide the molecular weight of the phycocyanin monomer experimentally. For this purpose, two methods were employed; sedimentation equilibrium measurements in 6 mol/l guanidine hydrochloride (GndCl) and the sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Moreover, the sedimentation coefficient and the intrinsic viscosity were measured in 6 mol/l GndCl.

Experimental

Protein Preparation. The crystalline phycocyanin from *Porphyra tenera* used in this study was isolated and purified as has been described previously.[4] The proper amount of GndCl was dissolved in phycocyanin phosphate buffer solution, the pH of which was 6.8 and with I value of 0.1. The solution was dialysed against a GndCl–phosphate buffer (pH 5.4, I 0.1) at 5 °C for 72 h. The concentration of GndCl in the external solution was then adjusted to be the same as that of the phycocyanin solution. The external solution was used as a solvent in this work.

Sedimentation Equilibrium Measurements. The sedimentation equilibrium experiments were performed with a Hitachi Model UCA-1 ultracentrifuge using interference optics. The rotor speed was set at 19780 rpm, and the temperature was maintained at 25.0 °C. All the runs were made at that speed for approximately 24 h so as to ensure that an equilibrium was established in a liquid column of 1.5 mm. Since the solution has a blue color, it was difficult to decide the fringe numbers at the cell bottom exactly. Therefore, the Nazarian method[5] was used for the determination of the molecular weight from sedimentation interference patterns. According to the Nazarian method, the apparent molecular weight, M^{a}, is given by the following equation:

$$M^{\mathrm{a}} = \frac{2RT}{(1-\bar{v}\rho)\omega^2} \frac{\mathrm{d} \ln \Delta_Q J}{\mathrm{d}(r^2)},$$

where R is the gas constant; T, the absolute temperature; $\bar{v}$, the partial specific volume of the solute; ρ, the density of the solvent; ω, the angular velocity of rotation, and r, the radial distance from the axis of rotation. $\Delta_Q J$ is the displacement of a fringe in the distance of the fixed Q and indicates the number of fringes crossed in traversing the interference pattern from r^2 to r^2+Q.

Sedimentation Velocity Measurements. The sedimentation velocity experiments were performed with the same ultracentrifuge as was used in the sedimentation equilibrium experiments, although Schlieren optics were used instead of the interference optics in those measurements. In all runs the rotor speed was set at 51200 rpm, and the temperature was maintained at 25.0 °C. The synthetic boundary cell was used, since the actual sedimentation rates of the solute were quite slow in 6 mol/l GndCl. The sedimentation coefficient, s, is represented by the following equation:

$$s = \frac{\ln (r_{\mathrm{p}}/r_{\mathrm{m}})}{\omega^2(t-t_0)},$$

where r_{p} is the boundary position measured from the second moment of the refractive index gradient; r_{m}, the position of the meniscus; t, the time, t_0, the initial time correction.

Partial Specific Volume. The partial specific volume of phycocyanin in 6 mol/l GndCl was calculated by the

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usual method from the densities of the solvent and solutions at 25.0 °C; using the solvent density of 1.1426 g/ml, the value of 0.746 ml/g was thus obtained. The densities were measured with a Shibayama Densimeter, SS-D-200 Ext. Type.

SDS-Polyacrylamide Gel Electrophoresis. The SDS-polyacrylamide gel electrophoresis was done by the Weber-Osborn procedure.[6] The phycocyanin sample was incubated at 37 °C for 2 h in a 0.01 M sodium phosphate buffer, 1% in SDS, and 1% in 2-mercaptoethanol. The gel buffer was a 0.1 M sodium phosphate–1% SDS solution. Electrophoresis was performed at a constant current of 8 mA per gel. The proteins used as the references were obtained from Boehringer Marnheim: cytochrom C (molecular weight $M=12500$), chymotrypsinogen A ($M=25000$), ovalbumin ($M=45000$), and bovine serum albumin ($M=67000$).

Viscosity Measurements. The viscosity measurements were made with an Ubbelohde dilution-type viscometer. Kinetic energy corrections were not made. The intrinsic viscosity was obtained by using Huggins' equation.

Results and Discussion

Figure 1 shows the absorption spectra of phycocyanin at various concentration of GndCl. From Fig. 1, the denaturation of phycocyanin may be considered to be complete in a 1 mol/l GndCl solution. On the basis of these results, the concentration of GndCl of 6 mol/l was used in the sedimentation measurements and the viscosity measurements in this work.

Figures 2 and 3 show the results of the sedimentation equilibrium measurements. Plot of ΔJ (the displacement of a fringe) *versus* r^2 is shown in Fig. 2. The $\Delta_Q J$'s were estimated by selecting the Q value of 1 cm^2; they are plotted against r^2 in Fig. 3. As is shown, the plot is linear, and the apparent molecular weight, M^a can be estimated from the slope of the plot. As Ohta and Kawahara[7] have pointed out, the monodispersity of the solute cannot be decided even if the Nazarian plot is linear, and the molecular weight thus calculated is the z-average molecular weight in the case of a polydisperse solute. In this work, the sample was assumed to be monodiperse in the denaturated solvent. The reciprocals of M^a thus obtained are plotted against the protein concentration, c, in Fig. 4. The true molecular weight of the phycocyanin monomer could be estimated by the linear extrapolation of the plot to infinite dilution; the molecular weight of 4.0×10^4 was obtained. In previous studies, the monomer molecular weight was assumed to be 4.2×10^4, a value arrived at on the basis of the results of the osmotic measurements performed at pH 5.4 and I 0.1; this value was about one-sixth of the molecular weight of the phycocyanin hexamer. The assumed value coincides with the value of 4.0×10^4 obtained in this work.

The results of the SDS–polyacrylamide gel electrophoresis are shown in Fig. 5. The mobility, R_f, was calculated by using the following equation:

$$R_f = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

The electrophoresis pattern of the phycocyanin sample shows two bands, the quantities of which are nearly equivalent. Several investigators have reported that the phycocyanin monomer consisted of two kinds of subunits. For example, Glazer and Fang[8] reported that the phycocyanin from a blue-green alga (*Synechococcus sp.*) was composed of an α subunit (molecular weight, 1.77×10^4) and a β subunit (molecular weight, 1.9×10^4). The molecular weights of the two kinds of

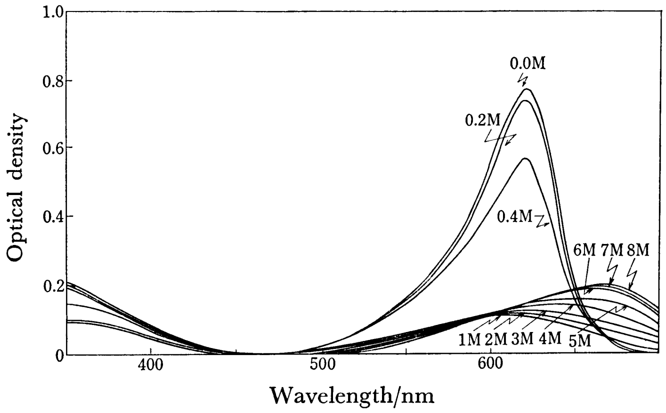

Fig. 1. The absorption spectra of phycocyanin at various concentrations of GndCl.

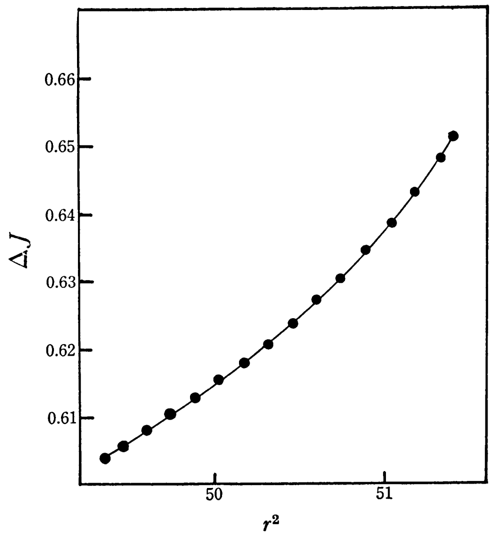

Fig. 2. A plot of ΔJ *versus* r^2, $c=0.0654$ g/dl.

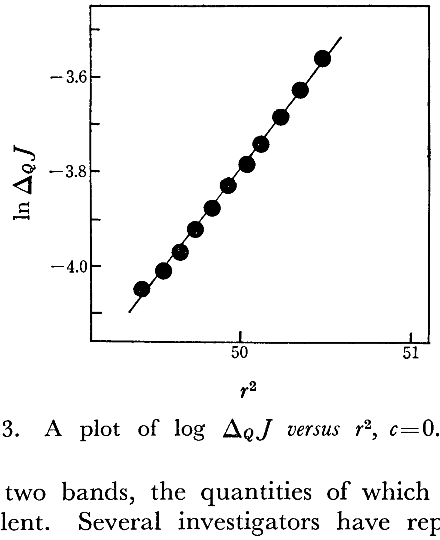

Fig. 3. A plot of log $\Delta_Q J$ *versus* r^2, $c=0.0654$ g/dl.

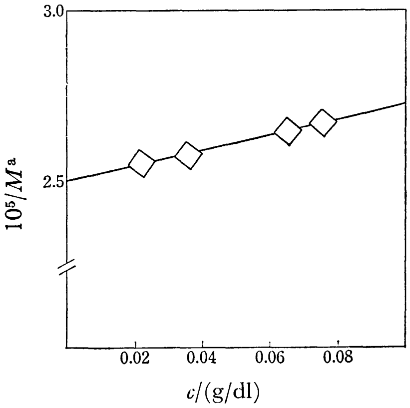

Fig. 4. A plot of $1/M^a$ *versus* c.

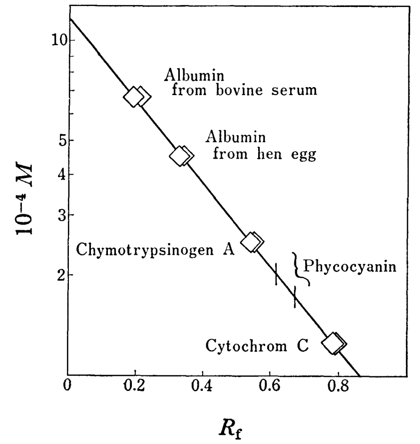

Fig. 5. A plot of M *versus* R_f.

subunits in this work were calculated to be 1.7×10^4 and 2.0×10^4 on the basis of the data in Fig. 5. Assuming that the phycocyanin monomer is composed of two polypeptide chains, the molecular weights of which are 1.7×10^4 and 2.0×10^4, the molecular weight of the monomer becomes 3.7×10^4. The value of 3.7×10^4 corresponds to the value of 4.0×10^4 obtained by the sedimentation equilibrium measurements. The results show that the assumption that the phycocyanin monomer is composed of the two subunits is reasonable. In view of the fact that the sedimentation equilibrium measurements form an absolute method for the determination of the molecular weight, the monomer molecular weight of the phycocyanin should be 4.0×10^4.

The hydrodynamic measurements, sedimentation velocity measurements and viscosity measurements will be described below.

All of our sedimentation velocity patterns showed a single peak. Figure 6 shows the sedimentation coefficient of phycocyanin as a function of the protein concentration. From Fig. 6, the values of the sedimentation coefficient at an infinite dilution, s_0, and the concentration-dependence coefficient, k_s, were determined to be 0.88 S (Svedberg unit) and 1.53 respectively. The value of $s_{25\text{w}}$, the s-value in water at 25 °C, was calculated to be 2.21 S by the usual method.

The viscosity data for phycocyanin in 6 mol/l GndCl at 25.0 °C are shown in Fig. 7. The intrinsic viscosity, $[\eta]$, was determined by the linear extrapolation of the plot of η_{sp}/c, where η_{sp} is the specific viscosity and where c is the concentration of phycocyanin. From this extrapolation, the values of $[\eta]$ and of the Huggins' constant, k', for the phycocyanin monomer in 6 mol/l GndCl at 25.0 °C were found to be 19.5 ml/g and 1.29 respectively.

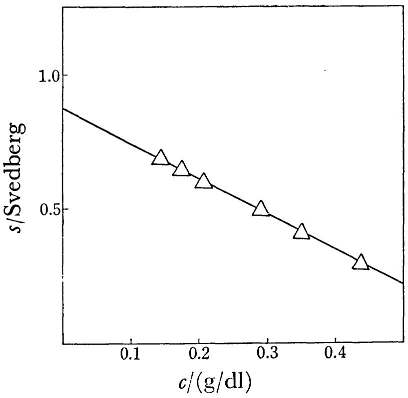

Fig. 6. A plot of s *versus* c.

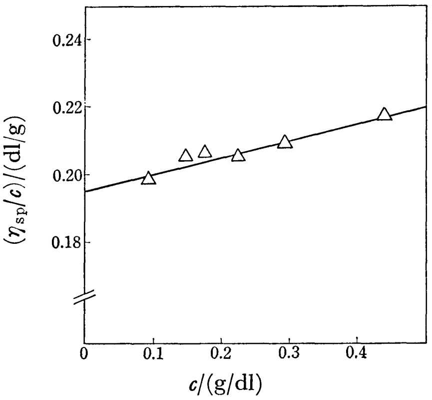

Fig. 7. A plot of η_{sp}/c *versus* c.

The Scheraga and Mandelkern parameter, β, was calculated by means of the following equation;

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

where N_A is the Avogadro number and η_0, the solvent viscosity (1.481×10^{-2} poise). The β-value of 2.6×10^6 was obtained for the phycocyanin monomer in 6 mol/l GndCl. Jennings[9] proposed a monomer model which was a prolate ellipsoid 35 Å in diameter and 105 Å in length. On the basis of this monomer model, the s- and $[\eta]$-values have been calculated to be 3.5 S and 4.2 ml/g respectively.[3] Using the s- and $[\eta]$-values, 2.0×10^6 is calculated for the β-value. The discrepancy of the β-values may be attributed to the fact that the conformation of the phycocyanin monomer in 6 mol/l GndCl was different from that of the monomer model proposed by Jennings; that is, the protein in 6 mol/l GndCl can be considered to be unfolded and to be expanded hydrodynamically.[10]

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