Solution Properties of Phycocyanin. III. Studies of the Sedimentation Equilibrium of Phycocyanin*

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(Received June 6, 1975)

The dissociation-association behavior of phycocyanin has been studied by sedimentation-equilibrium measurements under various pH and ionic-strength conditions. The weight-average molecular weight of phycocyanin changed according to the changes in concentration of the solute. From the concentration dependence of the molecular weight, it has been claimed that the dissociation-association equilibrium system of phycocyanin is trimer⇌monomer at pH 6.8 in the relatively low concentration region and hexamer⇌monomer at pH 5.4. However, the coexistence of the trimer⇌monomer and hexamer⇌monomer systems is in evidence in the concentration region larger than 0.06 g/dl. This dissociation-association behavior of phycocyanin is supported by the experimental results obtained from sedimentation-transportation[1] and osmotic-pressure measurements.[5]

In the course of our continuing study of the solution properties of phycocyanin, it was found, from an analysis of the sedimentation velocity data, that the dissociation-association reacting state for phycocyanin was a trimer⇌monomer system at pH 6.8 and a hexamer⇌monomer system at pH 5.4, and that the dissociation constants for the reacting systems were $0.13\sim0.15\times10^{-4}$ (g/dl)^2 for a trimer⇌monomer system at pH 6.8 and in the ionic-strength(μ) range of 0.1∼0.2, and $0.1\sim0.5\times10^{-13}$ (g/dl)^5 for a hexamer⇌monomer system at pH 5.4 and at an ionic strength of 0.1∼0.2. From the results, it was assumed that the predominant species of phycocyanin was a trimer at pH 6.8 and a hexamer at pH 5.4.[1] Since the dissociation constants described above were evaluated from the concentration dependence of the sedimentation coefficients, these constants should not considered to be thermodynamically the equillibrium values. Therefore, it is desirable to investigate the dissociation-association equilibrium of phycocyanin on the basis of sedimentation-equilibrium experiments.

The purpose of the work to be reported here is to determine the dissociation constant of phycocyanin under various conditions of pH and ionic strength by sedimentation-equilibrium measurements and to obtain information about the dissociation-association state of phycocyanin.

Experimental

Materials and Buffers. The crystalline phycocyanin used in this study was obtained from red algae *Porphyra tenera* by repeated precipitations with ammonium sulfate. The details of the purification of the protein were presented in an earlier paper.[1] Phosphate(pH 6.8, μ 0.1 and 0.2) and acetate (pH 5.4, μ 0.1 and 0.2) buffer solutions were used in this study.

Sedimentation-equilibrium Measurements. The sedimentation-equilibrium measurements were carried out using a magnetically suspended equilibrium-type ultracentrifuge with an interference optical system; a double sectorial cell was also used. The apparatus used here has been described in detail elsewhere.[2]** All the measurements were made at a given speed for approximately 24 hours so as to ensure that an equilibrium was established in an about 3-mm liquid column. The rotor speeds were set at approximately 6000∼9000 rpm, and the temperature was maintained at 25.00±0.01 °C through all the measurements.

The apparent weight-average molecular weight, M_w^{app}, and the apparent z-average molecular weight, M_z^{app}, of phycocyanin were calculated from Eq. (1) and Eq. (2).

$$M_w^{app} = \frac{2RT}{(1-\bar{v}\rho)\omega^2}\frac{1}{(r_b^2-r_a^2)}\frac{\Delta c}{c_0} \quad (1)$$

$$M_z^{app} = \frac{RT}{(1-\bar{v}\rho)w^2}\frac{(dc/dr)_b-(dc/dr)_a}{\Delta c} \quad (2)$$

In these expressions, $\bar{v}$ is the partial specific volume of the solute; ρ, the density of the solvent; ω, the angular velocity of the rotor; r, the radial position from the center of rotation in the ultracentrifuge cell, and r_a and r_b, the position of the meniscus and the cell bottom respectively. The concentration difference, Δc, between r_a and r_b, was calculated by the use of this formula:

$$\Delta c = (\Delta N)\lambda/(dn/dc)L$$

where ΔN is the number of fringes counted from the standard fringe along the direction of the ultracentrifugal field; λ, the wavelength of light (546 nm), dn/dc, the refractive-index increment, and L, the thickness of the cell (12 mm). The refractive index increment of the protein was obtained from the schlieren pattern of a protein solution of a known concentration; the value of 0.18 ml/g was obtained at 546 nm. This value for dn/dc was used throughout this study irrespective of the aggregation state of phycocyanin. For the partial specific volume, $\bar{v}$, of phycocyanin, the literature value[3] of 0.744 ml/g was used in all the measurements. The solvent density, ρ, was determined with a pycnometer approximately 2 ml in volume.

Determination of the Dissociation Constant. The determination of the dissociation constants for each dissociation-association system with a computational method was performed by using the sedimentation-equilibrium data. The computational method used in this study is essentially an extension of the theory for a monomer-dimer self-association system published by Williams.[4] In this procedure, it is assumed that all of the systems considered here are ideal solutions. The thermodynamic ideality of phycocyanin was confirmed by the experimental results from the osmotic-pressure measurements.[5]

For the i-mer⇌monomer equilibrium system, the weight-average molecular weight, M_w, is expressed by the following formula;

$$M_w = \frac{c_1M_1+c_iM_i}{c} \quad (3)$$

* Some of the experimental results in this paper were presented at the 23rd Annual Meeting of The Socity of Polymer Science, Japan, Tokyo, June 6, 1974.

** This apparatus is in the possession of the Department of Chemistry, Tokyo Kyoiku University.

where c is the total weight concentration of the solute, c_1 and M_1 are the weight concentration and the molecular weight of the monomer, and c_i and M_i are the weight concentration and the molecular weight of i-mer respectively.

The dissociation constant for the trimer$\rightleftharpoons$monomer system, K_3, is defined as Eq. (4) by using the mass-action law;

$$K_3 = c_1^3/c_3 \tag{4}$$

$$M_3 = 3M_1$$

$$c = c_1 + c_3 \tag{5}$$

From Eqs. (3)$\sim$(4), the following expression can be derived:

$$\frac{M_1^2(M_w^{app}-M_1)}{(3M_1-M_w^{app})^3} = \left(\frac{1}{4K_3}\right)c^2 \tag{6}$$

where M_w^{app} is the apparent weight-average molecular weight at the initial concentration, c. Equation (6) may be rewritten as Eq. (7):

$$\frac{(X-1)}{(3-X)^3} = \left(\frac{1}{4K_3}\right)c^2 \tag{7}$$

where X is M_w^{app}/M_1. It is clear that the quantity, $(X-1)/(3-X)^3$, becomes zero when $c=0$. Therefore, the dissociation, constant, K_3, can be determined from the initial slope of the plot of $(X-1)/(3-X)^3$ *versus* c^2.

By a similar treatment, Eq. (8) can be derived for the hexamer$\rightleftharpoons$monomer system by the same procedure as that used for Eq. (7):

$$\frac{(X-1)}{(6-X)^6} = \left(\frac{1}{625K_6}\right)c^5 \tag{8}$$

where K_6 is the dissociation onstant.

When the apparent z-average molecular weight, M_z^{app}, is used in place of M_w^{app} in Eqs. (7) and (8), the following equations, (9) and (10), are obtained:

$$\frac{(X-1)(4-X)^2}{(3-X)^3} = \left(\frac{27}{4K_3}\right)c^2 \tag{9}$$

$$\frac{(X-1)(7-X)^5}{(6-X)^6} = \left(\frac{6^6}{5^5K_6}\right)c^5 \tag{10}$$

where X is M_z^{app}/M_1.

Results and Discussion

The apparent weight-average molecular weight and the apparent z-average molecular weight were obtained from sedimentation-equilibrium measurements performed at a number of initial concentrations of the solute. Figure 1 shows the concentration dependence of the apparent weight- or number-average molecular weight of phycocyanin and, in the inset, that dependence in the low concentration region. The lines in this figure represent the theoretical curves except for (e). The (a), (b), (c) and (d) lines were calculated by using the dissociation constant obtained from the subsequent analysis in this work. The (a) and (b) lines were computed by using the dissociation constants obtained from the weight-average and the z-average molecular weight respectively for the trimer$\rightleftharpoons$monomer system, and the (c) and (d) lines, by using those constants for the hexamer$\rightleftharpoons$monomer system. The (e) line was drawn based on the dissociation constant for the hexamer$\rightleftharpoons$monomer system which was obtained from the sedimentation coefficient.[1]

As is shown in Fig. 1, the values for the apparent weight-average molecular weight depend on the change

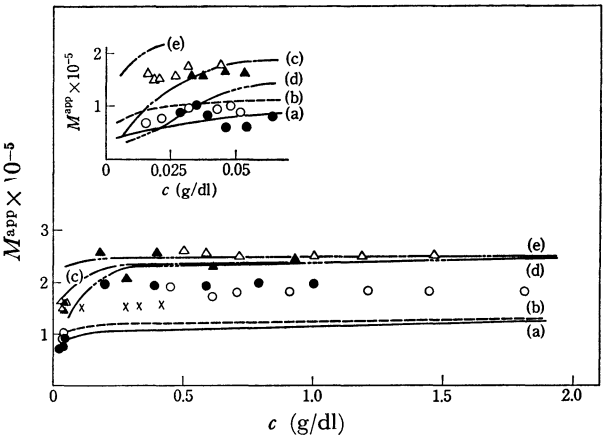

Fig. 1. Concentration dependence of apparent molecular weight of phycocyanin in various buffer solution over a wide concentration range; ○, pH 6.8 and $\mu=0.1$; ●, pH 6.8 and $\mu=0.2$; ×, pH 6.8 and $\mu=0.01$; △, pH 5.4 and $\mu=0.1$; ▲, pH 5.4 and $\mu=0.2$. Lines in this figure were obtained by using various K_3 and K_6 values; (a), $K_3=6.1\times10^{-4}$ (from M_w^{app}); (b), $K_3=1.4\times10^{-5}$ (from M_z^{app}); (c), $K_6=4.4\times10^{-10}$ (from M_w^{app}); (d), $K_6=2.4\times10^{-8}$ (from M_z^{app}); (e), $K_6=0.5\times10^{-13}$ (from s). These dissociation constants have units of $(g/dl)^2$ for K_3 and that of $(g/dl)^5$ for K_6.

in the pH of the solution. In addition, it is clear that the weight-average molecular weight in each system tends to decrease with a decrease in the concentration of the solute in the concentration region studied. The authors conclude from this evidence that phycocyanin undergoes a dissociation-association reaction in solution. The concentration dependencies of the apparent weight-average molecular weight under different pH values are independent of the ionic strength in the concentrations examined in this study. The mean values of the weight-average molecular weight were 78000 at pH 6.8 and 156000 at pH 5.4, while the values for the z-average molecular weight were 125000 at pH 6.8 and 181000 at pH 5.4.

It has been reported from the sedimentation-transportation experiment that the dissociation-association equilibrium system is trimer$\rightleftharpoons$monomer at pH 6.8 and hexamer$\rightleftharpoons$monomer at pH 5.4.[1] Therefore, these systems were assumed to be in a stable equilibrium state under each pH condition. The equilibrium constants were calculated from the experimental data by using Eqs. (7)$\sim$(10). The molecular weight of the monomeric species, M_1, is necessary for this calculation. As has been described in a previous paper[1], the phycocyanin molecule is stable in solution at pH 5.4, and the predominant species is the hexameric form under this pH condition. This conclusion receives support from the results of the osmotic-pressure measurement, which gives a value of 256000 as the number-average molecular weight of the protein.[5] It is, therefore, assumed that the molecular weight of the monomer is 42000.

Figure 2 shows the plot of the quantity, $(X-1)/(3-X)^2$, as a function of the square values of the concentration, c^2, where $X=M_w^{app}/M_1$. Since all of the

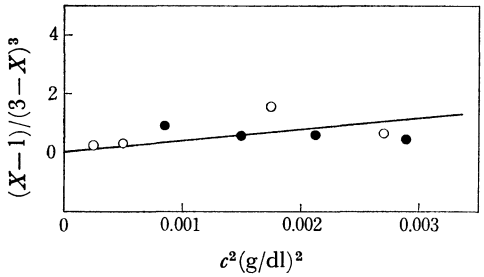

Fig. 2. Plot of $(X-1)/(3-X)^3$ with respect c^2 for phycocyanin in phosphate buffer solutions (pH 6.8); ○, $\mu=0.1$; ●, $\mu=0.2$. $X=M_w^{app}/M_1$.

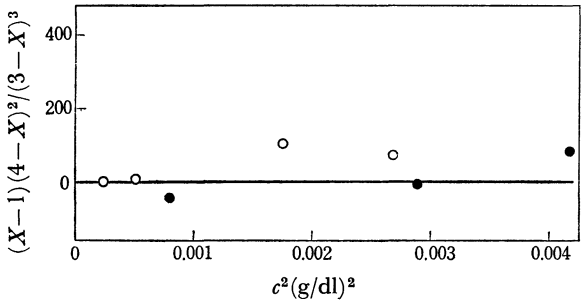

Fig. 3. Plot of $(X-1)(4-X)^2/(3-X)^3$ with respect to c^2 for phycocyanin in phosphate buffer solutions (pH 6.8); ○, $\mu=0.1$; ●, $\mu=0.2$. $X=M_z^{app}/M_1$.

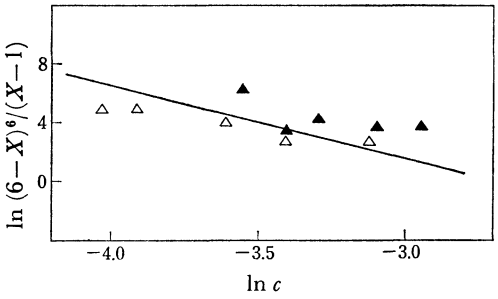

Fig. 4. Plot of $\ln(6-X)^6/(X-1)$ with respect to $\ln c$ for phycocyanin in acetate buffer solutions (pH 5.4); △, $\mu=0.1$; ▲, $\mu=0.2$. $X=M_w^{app}/M_1$.

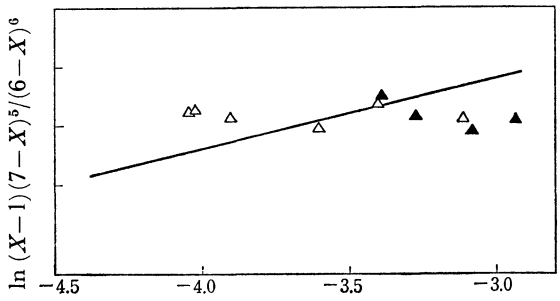

Fig. 5. Plot of $\ln(X-1)(7-X)^5/(6-X)^6$ with respect to $\ln c$ for phycocyanin in acetate buffer solutions (pH 5.4); △, $\mu=0.1$; ▲, $\mu=0.2$. $X=M_z^{app}/M_1$.

protein species in solution are expected to decompose to the monomer at an infinite dilution, the left-hand side of Eq. (7) approaches zero at an extremely low concentration. The dissociation constant, K_3, was calculated from the slope of the plot. Though there is some error in the z-average molecular weight as a result of the difficulty of making an accurate analysis of the experimental data, the dissociation constant, K_3, was calculated by using the M_z^{app} value as a trial (Fig. 3).

Figure 4 shows the plot of $\ln(6-X)^6/(X-1)$ *versus* $\ln c$. As is known from Eq. (8), the plot is a straight line with the value of slope -5. Although there are some scattering in the experimental data, the dissociation constant, K_6, was calculated from the interception of the ordinate for the straight line with the tangent value at $\ln c=0$, and was also calculated from M_z^{app} (Fig. 5).

The values for K_3 were given as 6.1×10^{-4} (g/dl)^2 from M_w^{app} and 1.4×10^{-5} (g/dl)^2 from M_z^{app}, while those for K_6 were 4.4×10^{-10} (g/dl)^5 from M_w^{app} and 2.4×10^{-8} (g/dl)^5 from M_z^{app}.

In order to ascertain the validity of the results obtained in this study, the concentration dependencies of M_w^{app} were recalculated from the K_3 and K_6 values obtained from the sedimentation-equilibrium measurement. There is another way to recalculate the concentration dependence of M_w^{app} by using the data obtained from the sedimentation-transportation method. The results obtained from these procedures are shown in Fig. 1. Although there is some difference between the numerical value for the dissociation constants obtained from the sedimentation equilibrium and those from the transportation measurements,[1] the concentration dependencies recalculated from the two procedures are essentially in accordance with the experimental results. In this figure, the molecular weights in the lower-concentration region are the weight-average values, while those at higher concentrations are the number-average values which were obtained from the osmotic-pressure experiments.[5]

As is shown in Fig. 1, the curves calculated by using the K_6 values at pH 5.4 agree with the experimental data over the whole range of concentrations. On the other hand, the curves calculated by using the K_3 values show a significant deviation from the experimental values in the concentration region larger than 0.06 g/dl. Therefore, it may be concluded from a comparison of the calculated and experimental values of M^{app} as functions of the concentration that the state of the dissociation-association equilibrium at pH 5.4 is hexamer⇌monomer over a wide concentration range. However, the equilibrium state at pH 6.8 depends on the concentration of the solute in the solution. The trimer⇌monomer system can be expected to be predominant in the concentration region less than 0.06 g/dl, and a higher-order aggregate, probably a hexamer, is formed in the concentration higher than 0.06 g/dl. It may be supposed both the trimer⇌monomer and hexamer⇌monomer systems exist in the higher-concentration region.

In the lower-concentration region, below 0.06 g/dl, the dissociation-association equilibrium is significantly shifted in one direction at both pH 6.8 and 5.4; that is, the predominant species at pH 6.8 are trimers, and those at pH 5.4, hexamers. Furthermore, the effect of the ionic strength on the equilibrium shift

in the higher-concentration region is almost negligible for the solution of pH 5.4. However, the effect of the ionic strength on the shift in the higher-concentration region is clear for the solution at pH 6.8.

The authors wish to express their gratitude to Professor Emeritus A. Kotera, of Tokyo Kyoiku University, for his interest and helpful discussion of the problem, and for permission to use the magnetically suspended equilibrium ultracentrifuge. They also wish to express their gratitude to Dr. K. Yoshizaki and Mr. K. Kanoo for their valuable assistance in the sedimentation-equilibrium measurements.

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