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Solution Properties of Phycocyanin. IV. Studies of the Self-association Equilibrium of Phycocyanin in a pH 6.8 Solution[*]

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The self-association of phycocyanin in a buffer of pH 6.8 with an ionic strength of 0.1 has been studied by sedimentation equilibrium measurements in the temperature range from 5.2 to 25 °C. The self-association of this system increases with a decrease in the temperature. A quantitative evaluation of the experimental data for this system indicates that the type of the association reaction is not a simple mechanism such as a monomer$\rightleftharpoons$*n*-mer, but the discrete monomer$\rightleftharpoons$trimer$\rightleftharpoons$tetramer mechanism is probable. Several thermodynamic quantities, such as the Gibbs free energy change, $\Delta G°$, the enthalpy change, $\Delta H°$, and the entropy change, $\Delta S°$, accompanying the self-association reaction were computed on the basis of the equilibrium constants. From the results, it is considered that the association process is similar to the ordinary crystallization, and that the formation of a tetramer proceeds at a higher concentration than that in which the trimerization reaction is completed.

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

In the course of our study of the solution properties of phycocyanin,[1—3] it was supposed, from an analysis of the sedimentation velocity data and of the sedimentation equilibrium data, that the self-association equilibrium was predominantly a monomer$\rightleftharpoons$hexamer at pH 5.4 and also probably a monomer$\rightleftharpoons$trimer at pH 6.8, both at the temperature of 25 °C. In the higher-concentration region at pH 6.8, however, the formation of the various aggregates higher than a trimer was probable, judging from the osmotic pressure measurements.

The purpose of the work reported here was to investigate the self-association reacting equilibrium of phycocyanin at pH 6.8 in more detail. That is, the equilibrium constants of the self-association of phycocyanin under various conditions of temperature were determined by sedimentation equilibrium measurements. The characteristic functions, such as the Gibbs free energy change, $\Delta G°$, the enthalpy change, $\Delta H°$, and the entropy change, $\Delta S°$, accompanying the self-association were evaluated on the basis of the equilibrium constants obtained.

Experimental

Materials. The crystalline phycocyanin used in this study was obtained from red alga *Porphyra tenera* by repeated precipitations with ammonium sulfate. The details of the purification of the protein were presented in an earlier paper.[1] The ionic strength was limited to 0.1 in the phosphate buffer in this work.

Sedimentation Equilibruim Measurements. The sedimentation equilibrium measurements were carried out using a magnetically suspended equilibrium-type ultracentrifuge and a Hitachi Model UCA-1 ultracentrifuge, with an interference optical system. All the measurements were made in about a 2-mm liquid column at a given speed for approximately 24 h so as to ensure that an equilibrium was established. The rotor speeds were set at approximately 8000—9000 rpm, and the measurements were done in the temperature range from 5.2 to 25.0 °C.

The apparent weight-average molecular weight at a position, r, in the solution column, $M_w^{app}(r)$, was calculated from the following equation:

$$M_w^{app}(r) = \frac{2RT}{(1-\bar{v}\rho)\omega^2}\frac{\mathrm{dln}\,c}{\mathrm{d}(r^2)}, \tag{1}$$

where $\bar{v}$ is the partial specific volume of the solute; ρ, the density of the solvent; ω, the angular velocity of the rotor; R, the gas constant, and T, the absolute temperature. The concentration, c, was calculated from the concentration difference, Δc, by the use of the formula

$$\Delta c = \Delta N\lambda/(\mathrm{d}n/\mathrm{d}c)L,$$

where ΔN is the number of fringes: λ, the wavelength of light (546 nm); dn/dc, the refractive-index increment, and L, the thickness of the cell (12 mm).

The Solvent Density and the Partial Specific Volume. The solvent density, ρ, at 16.5—25.0 °C was determined by means of a pycnometer approximately 10 ml in volume. The values of 1.0027 g/ml, 1.0039 g/ml, and 1.0046 g/ml were obtained at 25.0, 20.0, and 16.5 °C respectively. The values of 1.0052 g/ml at 11.5 °C and of 1.0056 g/ml at 5.2 °C were obtained by the linear extrapolation of the values at 16.5—25.0 °C.

Bull and Breese[4] have pointed out that the variation in the partial specific volume of the protein, $\bar{v}$, with the temperature is only *ca.* 0.08% at 5—25 °C. Accordingly, the literature value[5] of 0.744 ml/g was used for the partial specific volume of phycocyanin in all the measurements in this work.

The Refractive-index Increment. The refractive-index increment, dn/dc, was calculated from the schlieren pattern of a protein solution of a known concentration at 25.0 °C; this pattern has been obtained by using a synthetic-boundary cell. The value of 0.180 ml/g was obtained, and this value was used for the analysis of the experimental data in this work.

Results and Discussion

Figure 1 shows the concentration dependence of the apparent weight-average molecular weight of phycocyanin at pH 6.8, with the open and closed marks being indicative of the experiments performed at 5.2 and 25.0

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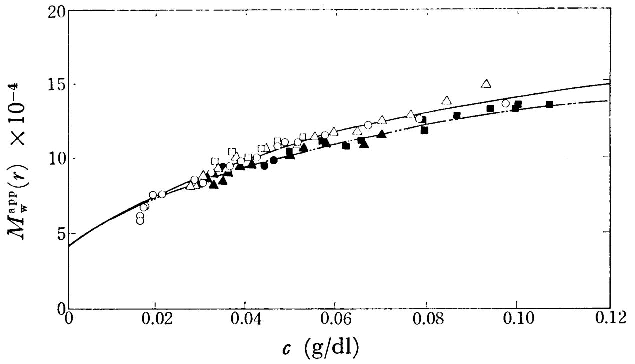

Fig. 1. Plots of $M_w^{app}(r)$ *versus* c for phycocyanin in a buffer of pH 6.8 and ionic strength 0.1 at 5.2 °C (upper curve) and at 25.0 °C (lower curve). The closed mark and the open mark correspond to the values at 25.0 °C and those at 5.2 °C, respectively. The various mark represent the values obtained with solutions of different initial concentration (g/dl): ○, 0.0048; △, 0.0054; □, 0.0031; ●, 0.0053; ▲, 0.0043; ■, 0.0084.

°C respectively. Similar results were obtained from the measurements performed at 20.0, 16.5, and 11.5 °C; these results stood between those at 25.0 °C and those at 5.2 °C in Fig. 1, in the order of the temperature. Consequently, it is clear that the association proceeds with a decrease in the temperature.

In our preceding paper,[3] the molecular weight of the monomer, M_1, of phycocyanin was assumed to be 42000 on the basis of the results of the osmotic pressure measurements. The value of 42000 is, then, used as the molecular weight of the monomer in the following analysis.

Tang and Adams[6] have proposed an analytical method applicable to any monomer⇌n-mer association system; by using it we can determine both the value of n and the equilibrium constant, K_n. According to their theory, the weight fraction of a monomer, f_1, is given as follows:

$$f_1=\frac{U+2-(2/n)-\{[U+2-(2/n)]^2-8(Un-1)/n\}^{1/2}}{4(n-1)/n}, \tag{2}$$

where

$$U=2(M_1/M_n^{app}(r))-(M_1/M_w^{app}(r))$$
$$=\frac{2}{c}\int_0^{c_1}\left(\frac{c}{c_1}\right)dc_1-\left(\frac{c}{c_1}\right)\frac{dc_1}{dc}, \tag{3}$$

$M_n^{app}(r)$ is the apparent number-average molecular weight at a position, r, which can be calculated from the value of $M_w^{app}(r)$ as measured by using the following equation:[7]

$$\frac{M_1}{M_n^{app}(r)}=\frac{1}{c}\int_0^c\frac{M_1}{M_w^{app}(r)}dc, \tag{4}$$

where c indicates the concentrations of all the species and where c_1 is the monomer concentration ($c_1=cf_1$). With the value of f_1, the following equation is derived:

$$(1-f_1)/f_1=K_n(cf_1)^{n-1}. \tag{5}$$

If the arbitrarily chosen value of n is suitable, a plot of $(1-f_1)$ *versus* $c^{n-1}f_1^n$ will give a straight line which passes through the point of orgin.

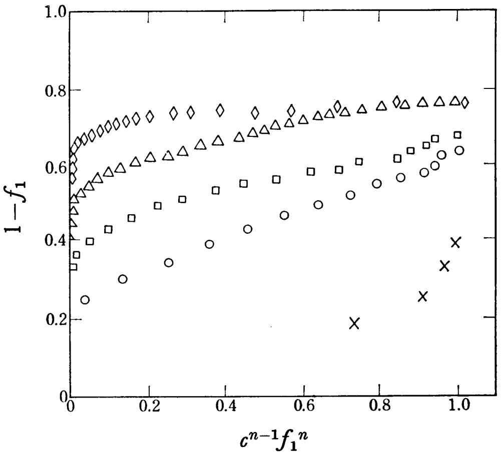

Fig. 2. Plots of $(1-f_1)$ *versus* $c^{n-1}f_1^n$, which are based on Eq. 5, for phycocyanin in a buffer of pH 6.8 and ionic strength 0.1 at 25.0 °C. ×, $n=2$; ○, $n=3$; □, $n=4$; △, $n=6$; ◇, $n=12$.

Their theory was applied to the self-association of phycocyanin in a buffer of pH 6.8 with ionic strength 0.1 at 5.2—25.0 °C. Figure 2 shows the plot of $(1-f_1)$ *versus* $c^{n-1}f_1^n$ at 25.0 °C, in which the n-values are chosen from 2 to 12. As is shown in Fig. 2, the plots were not straight lines passing through the point of origin; similar results were obtained at other temperatures. From these results, it may be concluded that the type of the association of phycocyanin in a buffer of pH 6.8 with an ionic strength 0.1 is not so simple as a monomer⇌n-mer.

In previous works[1,2] it was shown that the type of the association of phycocyanin at pH 6.8 was a monomer ⇌trimer at relatively low concentrations and that various higher aggregates were formed at higher concentrations. Therefore, we assume in this work that the type of the association of phycocyanin at this pH is a monomer⇌trimer⇌tetramer or a monomer⇌trimer⇌hexamer. Adams *et al.*[7,8] have analyzed the multi-component system and have proposed a procedure for evaluating an equilibrium constant for a monomer⇌dimer⇌trimer and a monomer⇌dimer⇌tetramer associations. Extending their treatment to a monomer ⇌trimer⇌tetramer, the following equation may be developed:

$$12M_1/M_w^{app}(r)=7+6f_ae^{-BM_1c}+6BM_1c$$
$$-1/[(M_1/M_w^{app}(r)-BM_1c], \tag{6}$$

where B is the non-ideal parameter. Moreover, the quantity f_a is the apparent weight fraction of the monomer and is obtained from the values of $M_w^{app}(r)$ and c according to the following equation:

$$\ln f_a=\int_0^c\left(\frac{M_1}{M_w^{app}(r)}-1\right)\frac{dc}{c}. \tag{7}$$

In this work, it is assumed that all of the systems considered here are pseudo-ideal solutions, since the thermodynamic pseudo-ideality of the phycocyanin solution was confirmed by the experimental results from the osmotic pressure measurements.[2]

When the non-ideal parameter, B, is zero, the following equation may be derived for a monomer⇌trimer

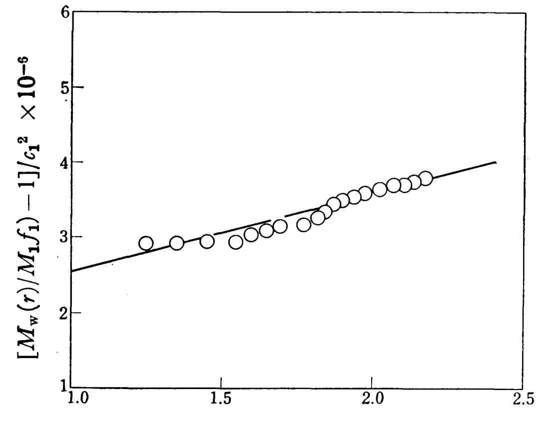

Fig. 3. Plot of $[(M_w(r)/M_1f_1)-1]/c_1^2$ *versus* c_1, which is based on Eq. 8, for phycocyanin in a buffer of pH 6.8 and ionic strength 0.1 at 25.0 °C.

⇌tetramer equilibrium:

$$[(M_w(r)/M_1f_1)-1]/c_1^2 = 3K_3 + 4K_4c_1, \tag{8}$$

where K_3 and K_4, which are calculated on the basis of the weight concentration (g/dl), are the equilibrium constants of a monomer⇌trimer equilibrium and a monomer⇌tetramer equilibrium respectively. According to Eq. 8, the plot of the value of the left-hand side of Eq. 8 *versus* c_1 will be linear. An example of such a plot is shown in Fig. 3, which is obtained from the data of the measurement at 25.0 °C. The plots at all the other temperatures were linear, like that at 25.0 °C, although they were excluded from Fig. 3 for the sake of simplicity. That is, all the plots of $[(M_w(r)/M_1f_1)-1]/c_1^2$ *versus* c_1 were linear. This means that the type of the association of phycocyanin in a buffer of pH 6.8 with an ionic strength 0.1 is probably a monomer⇌trimer⇌tetramer in the temperature range from 5.2 to 25.0 °C.

In the case of a linear plot such as that at 25.0 °C in Fig. 3, the values of K_3 and K_4 may be obtained from the intercept and the slope of the plot respectively. The values thus obtained are summarized in Table 1. These K_3- and K_4- values suggest that the lower aggregate, the trimer, decreases, while the higher aggregate, the tetramer, increases, with a decrease in the temperature. In order to ascertain the obtained values of K_3 and K_4, the values of $M_w(r)$ at some concentrations were calculated by using the K_3- and K_4-values and were compared with the values obtained experimentally. The calculated values were in good agreement with the experimental ones. Table 2 shows an example of the comparison.

A similar analytical procedure was applied to the experimental data assuming a monomer⇌trimer⇌hexamer system. The equilibrium constants of hexamerization, K_6, obtained decreased with a decrease in the temperature. The association proceeds with a decrease in the temperature, as is shown in Fig. 1. On the assumption that the higher aggregate may increase relatively with the process of association, the value of K_6 should increase with a decrease in the temperature. Thus, the associating mechanism of a monomer⇌trimer⇌hexamer system was not considered in this report.

Table 1. The equilibrium constants of the monomer ⇆ trimer and monomer ⇌ tetramer of phycocyanin at pH 6.8 and an ionic strength 0.1

T (°C)	$K_3 \times 10^{-4}$ (dl/g)^2	$K_4 \times 10^{-6}$ (dl/g)^3
5.2	0.36	0.25
11.5	0.34	0.28
16.5	0.45	0.38
20.0	0.75	0.18
25.0	0.68	0.18

Table 2. The comparison of the $M_w(r)$'s obtained at 25.0 °C and calculated by using K_3 and K_4

c (g/dl)	$M_w(r) \times 10^{-4}$	
	Obtained	Calculated
0	4.2_0	4.2_0
0.01	6.0_0	6.8_3
0.02	7.4_0	8.6_9
0.03	8.6_0	9.7_2
0.04	9.6_0	10.4
0.05	10.6	10.9
0.06	11.3	11.2
0.07	12.0	11.5
0.08	12.6	11.7
0.09	12.9	11.9
0.10	13.0	12.1
0.11	13.0	12.2
0.12	13.0	12.4

Figures 4 and 5 show the plots of ln k_3 *versus* $1/T$ and ln k_4 *versus* $1/T$ respectively, where k_3 and k_4 are the molar equilibrium constants. The values of enthalpy change, $\Delta H°$, accompanying the trimerization and the tetramerization were calculated to be 6.4 kcal/mol and −3.8 kcal/mol respectively from the slope in each plot. Table 3 lists the enthaply changes, $\Delta H°$, the Gibbs free energy changes, $\Delta G°$, and the entropy changes, $\Delta S°$, for both reactions.

From the negative value of $\Delta G°$ and the positive value of $\Delta S°$, it is clear that the aggregation process is spontaneous under the experimental conditions in this study. The Gibbs free energy changes in Table 3 correspond to −4——5 kcal/mol per monomer unit. The values are comparable to the values reported for many other proteins.[9] Oosawa and Asakura[10] have suggested that the aggregation process of protein is similar to a crystallization mechanism. Figure 6 shows the relation between the concentration of the aggregate and the total concentration of the solution, which is estimated by using the values of K_3 and K_4 here obtained. The behavior shown in Fig. 6 may be considered to be similar to crystalization. The estimation using the values of K_3 and K_4 shows that the c_4 becomes equal to c_3 when c=*ca.* 0.78 g/dl. In Fig. 6, the concentration range is limited to that used in the sedimentation equilibrium measurements. After that concentration the tetramer forms quickly,

Table 3. The characteristic thermodynamic functions accompanying the self-association reaction of phycocyanin

The units of ΔH° and ΔG° are (kcal/mol), and that of ΔS°, (cal/mol K)

	k (l/mol)$^{2 \text{ or } 3}$	ΔH°	ΔG°	ΔS°
monomer-trimer	(2.0—4.0) $\times 10^{10}$	6.4	−13.1—−14.4	69—70
monomer-tetramer	(4.5—9.3) $\times 10^{15}$	−3.8	−20.1—−21.3	59—60

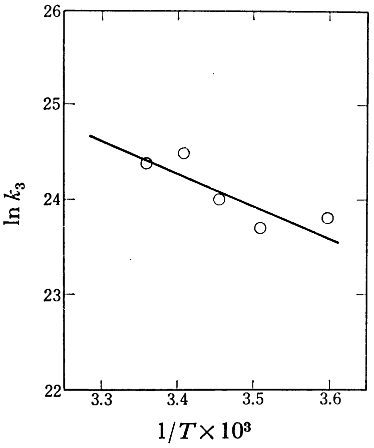

Fig. 4. Plot of ln k_3 *versus* $1/T$.

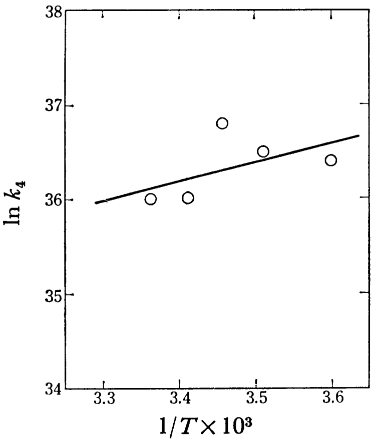

Fig. 5. Polt of ln k_4 *versus* $1/T$.

Although a large part of the aggregate is a trimer in concentrations under 0.1 g/dl at this pH and ionic strength, small amounts of a higher aggregate, which is assigned to a tetramer in this work, also exist in this system. That is the reason why we could not determine the molecular shape of phycocyanin at the pH and ionic strength of a previous work.[2] The number-average molecular weight, M_n of 1.7×10^5 was obtained in the concentration range from 0.5 to 1.5 g/dl from the osmotic pressure measurements.[2] The M_n is calculated to be 1.4×10^5 in the same concentration range by using the values of K_3 and K_4 here obtained. The calculated value of 1.4×10^5 is comparable to the experimental value of 1.7×10^5. Considering the facts that the phycocyanin molecule is most stable in solution at pH 5.4, and that the predominant species is assumed to be hexamer under this pH condition,[3] the associating system in this work may be intermediate from a monomeric state to a stable polymeric state, the hexamer. This is clear from the thermodynamic analysis of the association reaction of phycocyanin at pH 5.4.

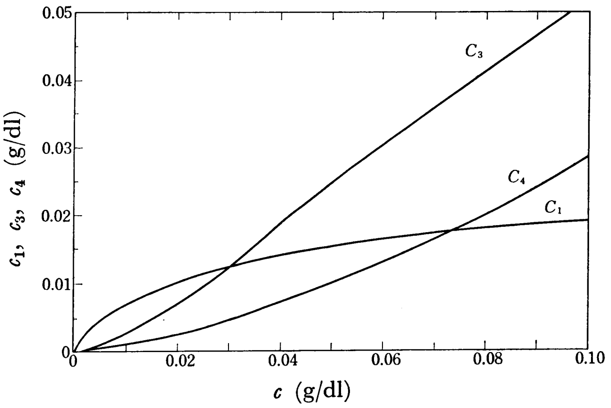

Fig. 6. The relation between the concentration of aggregate (c_3 and c_4) and the concentration of solution, which is estimated by using the values of K_3 and K_4 obtained.

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