Solution Properties of Phycocyanin. V. Studies of the Self-association Reaction of Phycocyanin in a pH 5.4 Solution†

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The self-association of phycocyanin in a buffer solution of pH 5.4 with an ionic strength of 0.1 has been studied by sedimentation equilibrium measurements in the temperature range from 5.0 to 25.0 °C. The thermodynamical treatment is also presented. The self-association of phycocyanin molecules proceeds with a decrease in the temperature. The concentration dependence of the apparent weight-average molecular weight of phycocyanin is interpreted in terms of a monomer⇌hexamer equilibrium system. It is clear from the thermodynamic parameters, ΔG^0, ΔH^0, and ΔS^0, that the self-association reaction is spontaneous in the solution of pH 5.4. In the association process, the formation of hydrophobic bonds is suggested by the large positive entropy change accompanying association reaction of phycocyanin molecules.

The dissociation-association properties of phycocyanin have been studied by many investigators. Some investigations in regard to the dissociation-association reaction system have also been reported in publications from this laboratory. Especially, it was suggested that the dissociation-association reacting state was precisely a monomer⇌hexamer system at pH 5.4, and also a monomer⇌trimer at pH 6.8 in relatively low concentrations.[1,2] In the higher concentration region, however, the existence of various aggregates higher than trimer was presumed from the osmotic pressure measurements under pH 6.8. The molecular weight of phycocyanin has been obtained as 2.56×10^5 from the osmotic pressure measurements at pH 5.4. The value was considered to be the molecular weight of the higher aggregate(hexamer) of phycocyanin.[3] The electron micrographic pattern of phycocyanin showed that the phycocyanin molecule was homogeneous and quite stable in the pH region 4.0—5.6, while, in contrast, it was unstable at a higher pH region.[4]

In the previous paper,[5] we postulated, from the results of the sedimentation equilibrium measurements under various temperature conditions at pH 6.8 and at the ionic strength of 0.1, that the dissociation-association behavior of phycocyanin could be explained by assuming a monomer⇌trimer⇌tetramer equilibrium system at this pH and ionic strength. Moreover, it was suggested from an analysis of the thermodynamic parameters, such as the Gibbs free energy change, ΔG^0, the enthalpy change, ΔH^0, and the entropy change, ΔS^0, that the associating reaction was analogous to the ordinary crystallization.

This work was undertaken in order to study the dissociation-association behavior of phycocyanin in solution at pH 5.4 and at the ionic strength of 0.1. In order to determine the equilibrium constants of phycocyanin under various temperature conditions, sedimentation equilibrium measurements in an acetate buffer solution at the ionic strength of 0.1 were performed at 5.0, 15.0, 20.0, and 25.0 °C. From the thermodynamic quantities, the ΔG^0, ΔH^0, ΔS^0 values, accompanied with the dissociation-association reaction, were estimated, and the mechanism of the dissociation-association of phycocyanin was discussed based on these quantities.

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Experimental

Protein Preparation. The crystalline phycocyanin from *Porphyra tenera* used in this study was isolated and purified as has been described previously.[1] The acetate buffer solution was used as a solvent, and the ionic strength was limited to 0.1. The ionic strength was the same as that previously used in the sedimentation equilibrium measurements performed at pH 6.8. The purity of the phycocyanin was ascertained from the measurements of the absorbance at 620 nm and at 280 nm in a phosphate buffer solution. The ratio of absorbance at 620 nm to that at 280 nm has usually been used as a criterion for judging the purity of phycocyanin; when the ratio is greater than 4.0, the protein has been considered to have a high purity.[6] The value of the ratio measured was greater than 4.7 for the phycocyanin obtained in this work. Therefore, the phycocyanin used in this study was a highly purified sample.

Sedimentation Equilibrium Measurements. Sedimentation equilibrium experiments were performed on the phycocyanin-buffer solutions at 5.0, 10.0, 20.0, and 25.0 °C respectively, using a Hitachi Model UCA-1 ultracentrifuge with an interference optics. The rotor speeds were set at approximately 8000—9000 rpm. All the runs were made at a given speed for approximately 24 h so as to ensure that an equilibrium was established in about a 2-mm liquid column. The apparent weight-average molecular weights at several radial positions in the solution column were evaluated by the method reported previously.[5] The solvent densities at 15.0, 20.0, and 25.0 °C were deterined in a 10-ml pycnometer. Since it was difficult to determine the solvent densities at low temperatures, the densities at 5.0 and 10.0 °C were estimated by the linear extrapolation of the values of the densities at 15.0, 20.0, and 25.0 °C repectively. By the same method, the solution densities at 5.0 and 10.0 °C were estimated. The partial specific volume of phycocyanin, $\bar{v}$, was calculated by the usual method from the densities of the solvents and solutions at 25.0 °C; the value of 0.749 ml/g was thus obtained. The variation in the partial specific volume with the temperature was very small;[7] therefore, the value of 0.749 ml/g was used in all the calculations of the molecular weight of phycocyanin in this work. This value is in good agreement with that of 0.744 ml/g(pH 5.3—7.0) reported by Hattori *et al.*[8]

Results and Discussion

Figure 1 shows a plot of the apparent weight-average molecular weight *versus* the concentration of protein in the solution column of the ultracentrifuge cell for the sedimentation experiment at 25.0 °C. As shown in this figure, the apparent weight-average molecular weights increase with an increase in the solute concentration in the low concentration region, and the molecular weight reaches a saturated value in the higher concentration region. This behavior is characteristic of a self-association system. Similar results were obtained from the measurements performed under the other temperature conditions; the saturated values of the molecular weight in the higher concentrations obtained from these measurements increased slightly as the temperature fell.

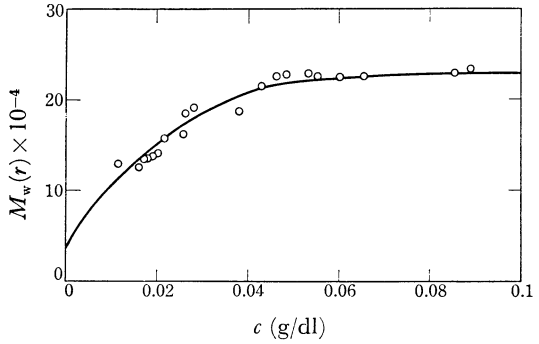

Fig. 1. Plot of apparent weight-average molecular weight as a function of concentration for phycocyanin solution at pH 5.4, μ=0.1, and 25 °C.

In the equilibrium mixture system, the apparent weight fraction of the monomer, f_a, is related to $M_w^{app}(r)$ as follows:[9]

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

where

$$\ln f_a = \ln f_1 + BM_1c. \quad (2)$$

In these relations, $M_w^{app}(r)$ represents the apparent weight-average molecular weight at a radial position, r, in the sedimentation equilibrium cell; M_1 is the molecular weight of the monomer; f_1, the weight fraction of the monomer, and B, the non-ideal parameter. The value for M_1 was assumed to be 4.20×10^4 from the previous result of the osmotic pressure measurements performed at pH 5.4, which was about one-sixth of the molecular weight of the phycocyanin hexamer.[3] It becomes clear from previous works that the phycocyanin solution may be considered to be a pseudo-ideal solution.[3,5] Therefore, the non-ideal parameter, B, in relation (2) may be neglected, Therefore, f_a can be replaced by f_1. Once f_1 is determined, the concentration of the monomer in solution, c_1, is obtained from the following relation:

$$c_1 = c\cdot f_1. \quad (3)$$

In the monomer⇌n-mer type of self-association, the concentration of n-mer, c_n, is expressed as follows:

$$c_n = c - c_1. \quad (4)$$

This relation can be rewritten as

$$c - c_1 = K_n\cdot c_1^n, \quad (5)$$

where K_n represents the equilibrium constant on the weight-concentration scale of the monomer⇌n-mer system. If the value of n arbitrarily chosen is suitable, the plot of $(c-c_1)$ versus c_1^n will give a straight line which passes through the point of origin. In this study, such a plot could be obtained over the whole temperature range when the n-value was assumed to be 6, as is shown in Fig. 2. It may be concluded from the result described just above that the type of self-association of phycocyanin used in this study is a monomer⇌hexamer system at pH 5.4.

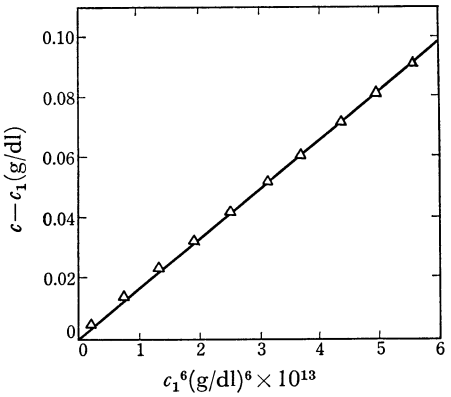

Fig. 2. Plot of $c-c_1$ *versus* c_1^6. The slope of this plot gives the equilibrium constant, K_6.

Table 1. Equilibrium constants at different temperatures

Temp (°C)	K_6(dl/g)^5	k_6(1/mol)^5
25.0	2×10^{11}	4×10^{28}
20.0	6×10^{11}	1×10^{29}
15.0	5×10^{11}	1×10^{29}
10.0	10×10^{11}	2×10^{29}
5.0	23×10^{11}	5×10^{29}

The molar equilibrium constant, k_6(1/mol)^5, was calculated by means of this relation: $k_6=\frac{1}{6}\cdot\left(\frac{M_1}{10}\right)^5\cdot K_6$.

Figure 2 shows the plot of $(c-c_1)$ *versus* c_1^6 at 25.0 °C. The equilibrium constant, K_6, can be evaluated from the slope of such a plot. Thus, the equilibrium constants at 5.0, 10.0, 20.0, and 25.0 °C were obtained. These values are summarized in Table 1. From the value of K_6, it is reasonable to say that the self-associating reaction of phycocyanin is practically hexamerization at pH 5.4 and the ionic strength of 0.1; this tendency becomes more outstanding with the decrease in the temperature. As is shown in Fig. 3, the characteristic dependence of the self-association on the temperature is clear from the van't Hoff plot, which is the relationship between the logarithm of the equilibrium constant on the molar scale and the reciprocal of the absolute temperature. The value of the equilibrium constant on the molar scale, k_6, is calculated from that on the weight-concentration scale by using the following relation:

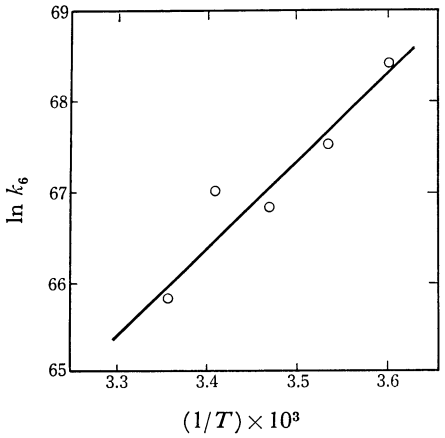

Fig. 3. The van't Hoff plot, $\ln k_6$ *versus* $1/T$, for the phycocyanin systems at five temperatures. The slope of this plot, calculated by a linear regression analysis, is $-\Delta H^0/R$.

$$k_6 = \frac{1}{6}\cdot\left(\frac{M_1}{10}\right)^5\cdot K_6 .$$

The ΔH^0 is evaluated as -19 kcal/mol from the van't Hoff plot, and the association reaction of phycocyanin is exothermic. It was concluded in a previous report[5] that the association reaction of phycocyanin at pH 6.8 was exothermic and that the value of ΔH^0 for the monomer⇌tetramer was -3.8 kcal/mol. Therefore, the difference in the enthalpy change shows that, from the viewpoint of energetics, the polymerization to a hexamer at pH 5.4 is more favorable than that to a tetramer at pH 6.8.

Table 2. Thermodynamic parameters at different temperatures

Temp (°C)	ΔG^0 (kcal/mol)	ΔS^0 (cal/K mol)
25.0	−39	67
20.0	−39	68
15.0	−38	66
10.0	−38	67
5.0	−38	68

$\Delta H^0 = -19$(kcal/mol).

The Gibbs free energy change, ΔG^0, and the entropy change, ΔS^0, accompanied by the dissociation-association of phycocyanin, can be calculated by using the following relations:

$$\Delta G^0 = -RT\ln K, \tag{6}$$

$$\Delta S^0 = \frac{\Delta H^0 - \Delta G^0}{T}. \tag{7}$$

The ΔG^0 and ΔS^0 values are calculated to be -38——-39 kcal/mol and 66—68 cal/K mol respectively. These values are listed in Table 2. If the aggregate at pH 5.4 is assumed to be a hexamer, the ΔG^0 value for the monomer unit is evaluated to be -6.5 kcal/mol from one-sixth of ΔG^0 for the hexamer. This result is not so different from the value of ΔG^0 reported for the monomer unit of phycocyanin at pH 6.8 (-5.0——-5.3 kcal/mol).[5]

In other proteins, similar values have been reported for the ΔG^0 — for example, α-amylase (pH 7, 20 °C), -6.3 kcal/mol, and hemerythrine (pH 7, 5 °C), -5.8 kcal/mol.[10]

In the previous report,[5] the value of ΔS^0 for the monomer⇌tetramer at pH 6.8 was calculated to be 59—60 kcal/K mol. The value of ΔS^0 for the monomer ⇌hexamer at pH 5.4 in this study is 66—68 cal/K mol, almost the same order as that for the monomer⇌ tetramer.

From the negative values of ΔH^0 and ΔG^0 and the positive value of ΔS^0 for the phycocyanin aggregation obtained in this study, it is clear that the self-association reaction of phycocyanin is spontaneous under the solution conditions of pH 5.4 and an ionic strength of 0.1.

In the association reaction, the loss in entropy is expected to be quite high because of the increase in the restriction for the motion of molecules. However, the value of the entropy change of the phycocyanin obtained in this work is a large positive one. Scherga and his co-workers have studied the thermodynamic properties of hydrophobic bonds in protein.[11–13] They suggested that the positive entropy change accompanying the association reaction of protein is due to the changes in the water structure around the side chain which are caused by the formation of hydrophobic bonds, and that the hydrophobic bonds can contribute to the stabilization of protein structures. Cann has observed a large entropy change caused by the antigen-antibody reaction, and suggested that it is a result of the release of bound water from antigen and antibody molecules.[14]

As described above, the formation of a hydrophobic bond produces a reduction of the water in contact with the protein molecules and the destruction of that structure, and the freedom of water molecules increases. This brings about an increase in the entropy of the reaction system. The positive changes of entropy accompnying the association are currently interpreted as being due to the formation of a hydrophobic bond between monomers and also to the change in the water structure around a polymer. Scott and Berns have suggested that the hydrophobic bonding force and electrostatic force are involved in the interaction of phycocyanin molecules.[15] MacColl *et al.* have reported that the phycocyanin hexamer appears to be more stabilized by hydrophobic force than the trimer.[16]

Although it does not necessarily mean that the positive change of entropy is wholly attributable to the formation of the hydrophobic bond and the change in the water structure, the large value of ΔS^0 observed in this study suggests a change in conformation, with the cooperative formation of many bonds between monomers.

Nagasawa and Holtzer proposed an interesting method for the estimation of the non-electrostatic part of the standard free energy change accompanying the dissociation reaction of protein from the experimental potentiometric curve.[17] Their method will be of great help in attacking the aggregation mechanism of phycocyanin.

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