An Ultracentrifuge Study of C-Phycocyanin Aggregation†

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ABSTRACT: The molecular weight of C-phycocyanin has been determined as a function of protein concentration in a sodium acetate buffer solution of pH 4.8 at 21.2 °C by the Yphantis method. The higher aggregate, the dodecamer (19 S), is first removed by dissociation simply by dialysis against pH 3.9 acetate buffer solution. The molecular weight data are best interpreted by the simultaneous presence of monomers, trimers, and hexamers. Assuming a monomer $\overset{K_{13}}{\rightleftharpoons}$ trimer $\overset{K_{36}}{\rightleftharpoons}$ hexamer equilibrium system, the equilibrium constants have been calculated, and the values are $K_{13} = 1.4 \times 10^{12}$ (L/mol)^2 and $K_{36} = 6.1 \times 10^5$ L/mol. The corresponding free energies for each step have been calculated to be −16.3 kcal/mol ($\Delta G°_{13}$) and −7.76 kcal/mol ($\Delta G°_{36}$). With these values of K_{13} and K_{36}, the weight-average molecular weight as a function of concentration is calculated. Satisfactory agreement is obtained between the calculated curve and the experimental data. Sedimentation velocity studies performed with a band-forming centerpiece, on the same protein solutions used for the sedimentation equilibrium studies, confirm the presence of three species: monomers, trimers, and hexamers.

The aggregation properties of phycocyanin have been studied by many investigators (Scott & Berns, 1965; Saito et al., 1974; Berns & Morgenstern, 1966) and are of central importance to understanding phycobilisome formation and stability (Gantt, 1975; Gantt et al., 1976). The monomer, trimer, hexamer, and dodecamer species are present in varying concentration as a function of pH (MacColl et al., 1971b; Kao et al., 1971). MacColl et al. (1971a) demonstrated that a solution of C-phycocyanin from *Phormidium luridum* at pH 3.9 contained only the monomer (3S) species. Kao et al. (1971) indicated that at the protein's isoelectric point, pH 4.7, a mixture of monomer (3 S), trimer (6 S), hexamer (11 S), and dodecamer (19 S) was present. Recently, Saito et al. (1978) suggested that the process, monomer ⇌ hexamer, explains the self-association of phycocyanin in a pH 5.4 solution. The equilibrium constants as well as thermodynamic parameters were calculated. Previous studies provided the association constant of the monomer ⇌ hexamer reaction (MacColl et al., 1971a,b; Saito et al., 1978). In the present work, in anticipation of careful kinetic experiments on the aggregation process of C-phycocyanin, we have deduced that close to the isoelectric point, at pH 4.8, phycocyanin is present as an equilibrium mixture containing monomers, trimers, and hexamers. The equilibrium constants of the monomer ⇌ trimer and the trimer ⇌ hexamer reactions are determined from careful sedimentation equilibrium experiments. This is the first step in a quantitative study of the equilibrium and kinetic self-aggregation properties of C-phycocyanin.

Experimental Procedures

Preparation of Phycocyanin Solution. C-Phycocyanin from the blue-green alga, *P. luridium*, was obtained and purified as described previously (MacColl et al., 1971a). The purified protein was stored under 50% saturated ammonium sulfate at 4 °C. For sedimentation equilibrium studies, aliquots of this solution were removed and spun down as needed. The precipitate was dissolved in a sodium acetate buffer of pH 3.9 at an ionic strength of 0.1. This phycocyanin solution was then dialyzed into the pH 3.9 acetate buffer for 24 h in the cold room (4 °C) with two changes of the buffer solution. This protein solution was then mixed with an equal volume of 0.3 M NaOH to bring the pH to 4.8. For sedimentation velocity studies, three different preparations of phycocyanin solution at pH 4.8 were used. For preparation I, the lyophilized phycocyanin was dissolved in sodium acetate buffer, pH 4.8, at an ionic strength of 0.1. For preparation II, the lyophilized phycocyanin was dissolved and dialyzed in pH 3.9 acetate buffer overnight, and then the solution was dialyzed in pH 4.8 acetate buffer. Preparation III was identical with the solutions prepared for the sedimentation equilibrium studies. The protein samples were clarified by sedimentation at 48200*g* for 30 min just before performing the experiments. Reagent-grade chemicals were used. Protein concentrations were determined by a Gilford 2400 spectrophotometer and Perkin-Elmer 320 spectrophotometer. The value of 6.57 was used for the specific absorption of the monomer for a 1 mg/mL solution in a 1-cm cell at a wavelength of 615 nm (Glazer, 1976). The protein concentration of the pH 3.9 solution was first determined. After mixing the solution with an equal volume of 0.3 M NaOH, we obtained the OD value at 280 nm. The slope from a plot of OD_{280nm} vs. protein concentration gives the extinction coefficient (ϵ) of phycocyanin in pH 4.8 acetate buffer solution and the value is 1.861.

Sedimentation Velocity Studies. C-Phycocyanin solutions at pH 4.8 were studied by sedimentation velocity in the Spinco Model E ultracentrifuge with absorption and Schlieren optics. For these experiments an AN-D rotor was used at the rotor speed of 60 000 rpm, and the Schlieren pattern was recorded with Kodak spectroscopic type IN photographic plates. Low concentration sedimentation velocity data was collected by using the photoelectric scanner and recorder with the monochromator at several wavelengths (520, 540, 560, and 580 nm).

Ultracentrifuge Studies with the Band-Forming Centerpiece. The sedimentation velocity studies of phycocyanin were also performed with a double-sector analytical band-forming centerpiece (Vinograd et al., 1963) together with the photoelectric scanning system in the Spinco Model E ultracentrifuge. In these experiments 10 μL of phycocyanin solution, containing between 0.16 and 0.66 mg/mL, was layered on to a bulk solution of 5% sucrose in sodium acetate buffer, pH 4.8. Sedimentation coefficients determined in 5% sucrose were corrected to correspond to water at 20 °C as described by Lee & Berns (1968b).

Equilibrium Ultracentrifugation. All experiments were performed at 21.2 °C, using a Spinco Model E analytical ultracentrifuge equipped with electronic scanner, monochro-

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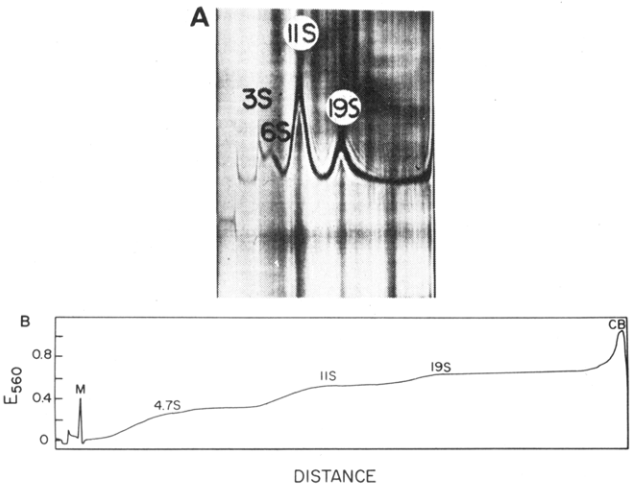

FIGURE 1: (A) Typical Schlieren pattern for a solution of C-phycocyanin (12.4 mg/mL) at pH 4.8 (preparation I). The picture was taken at 20 min after full speed (ω = 60 000 rpm) was reached. (B) Sedimentation pattern for a solution of C-phycocyanin (0.33 mg/mL; preparation I) with the photoelectric scanning system on the Spinco Model E ultracentrifuge. Light of wavelength 560 nm was used. The time after full speed (60 000 rpm) was reached was 25 min. M, location of meniscus; CB, location of cell bottom.

mator, multiplexer, and a temperature-regulation unit. The temperature of 21.2 °C was chosen because it is close to room temperature. The solutions were prepared by mixing an equal volumes of phycocyanin at pH 3.9 and 0.3 M NaOH. The AN-G rotor with a double-sector centrifuge cell and the absorption optics were used. About 4 drops of carbon tetrachloride was added to the centrifuge cell to form the false bottom. The cell compartments were loaded by means of a Hamilton gas-tight syringe with 100 μL of protein solution and 120 μL of solvent to give a solution column height of approximately 3.0 mm. The rotor speed of 20 000 rpm was chosen according to the method of Yphantis (1964). The equilibrium was achieved after 24 h. The value of 0.75 (Scott & Berns, 1965; Scott, 1965) was used for the partial specific volume of protein. Polynomial regression analysis of the data, where appropriate, was performed using a PDP 1145 computer, while simpler regression analysis programs were performed on an Apple II plus laboratory computer.

Results and Calculations

Ultracentrifuge Studies with Schlieren and Absorption Optics. The phycocyanin purified for this study had different aggregation properties depending on the final treatment of the sample prior to sedimentation. For the sample of preparation I, in which the sample was simply dissolved in pH 4.8 acetate buffer and then centrifuged, four boundaries (3 S, 6 S, 11 S, and 19 S) were observed in the Schlieren sedimentation velocity patterns (Figure 1a). At lower concentration (0.33 mg/mL) at least three boundaries (4.7 S, 11 S, and 19 S) were observed with absorption optics (Figure 1B). The sedimentation coefficient of 4.7 S for the monomer species seems too high for monomer alone and is probably a combination of 3S and 6S species. A completely different Schlieren pattern was observed if the protein sample was dialyzed at pH 3.9 first and then dialyzed into pH 4.8 acetate buffer (preparation II). Figure 2 indicated the coexistence of monomers and hexamers. In some cases, a very small boundary was observed that corresponded to a 6S species. No 19 S was obtained. The result from sedimentation velocity experiments for phycocyanin solution under preparation III, when the pH 3.9 protein solution is brought to pH 4.8 by addition of 0.3 M NaOH, is

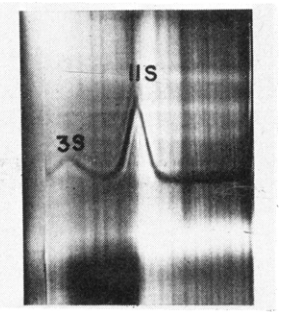

FIGURE 2: A Schlieren pattern for a solution of C-phycocyanin (8.82 mg/mL) at pH 4.8 (preparation II). The time was 31 min after full speed was reached.

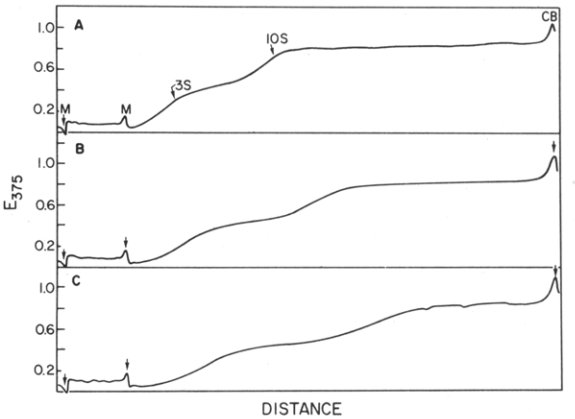

FIGURE 3: Sedimentation patterns as a function of time for a solution of C-phycocyanin (0.847 mg/mL) from *P. luridum* (preparation III). Light of wavelength 375 nm was used for all scans. The speed was 60 000 rpm at room temperature. The times after full speed was reached were (A) 1055, (B) 1640, and (C) 2271 s.

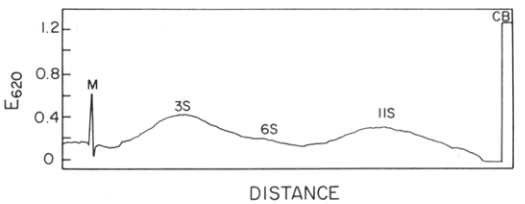

FIGURE 4: Sedimentation pattern for a solution of C-phycocyanin (0.37 mg/mL; preparation III) with the photoelectric scanner and band-forming centerpiece. Scans were made at 620 nm at 60 000 rpm. The time after full speed was reached was 2420 s.

shown in Figure 3. There is no plateau region found between the monomer and hexamer boundaries, and the 19 S species is absent.

Ultracentrifuge Studies with the Band-Forming Centerpiece. Sedimentation velocity studies with absorption optics at 620 nm were performed utilizing a band-forming centerpiece. In Figure 4 three bands were seen when a phycocyanin solution (preparation III) was placed on a 5% sucrose column at pH 4.8. The $s^0_{20,w}$ values for the fastest and slowest sedimenting bands were 10.4 S and 2.4 S. The band between the monomer and the hexamer species is apparently the trimer species.

Equilibrium Ultracentrifugation. Sedimentation velocity studies clearly demonstrate that the species present in pH 4.8 C-phycocyanin solution is strongly dependent on the prior treatment of the sample. The phycocyanin solutions of prepration III were used for the sedimentation equilibrium studies since these conditions can easily be used in later stopped-flow kinetic experiments. Figure 5 gives a plot of the weight-average molecular weight of C-phycocyanin as a function of optical density, which is linearly related to protein concentration. In this plot we have employed the method of

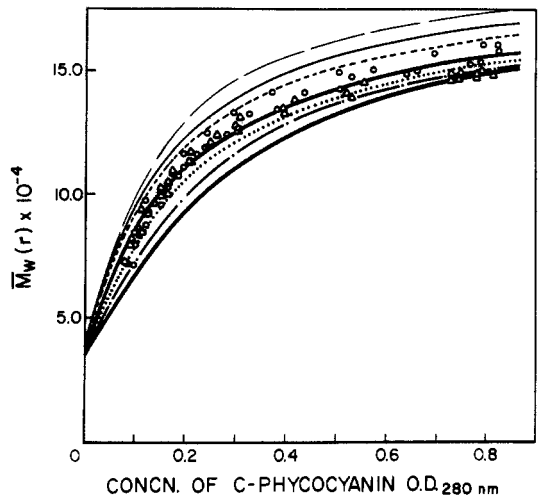

FIGURE 5: Experimental weight-average molecular weight vs. optical density of C-phycocyanin at 280 nm (preparation III): (○) values from experiments with a starting concentration of 0.101 mg/mL and (△) values from experiments with a starting concentration of 0.133 mg/mL, for the monomer–trimer–hexamer reaction as outlined in the text. (---) Calculated for $K''_{13} = 300$ and $K''_{36} = 26.11$; (—) (upper) calculated for $K''_{13} = 400$ and $K''_{36} = 14.7$; (---) calculated for $K''_{13} = 500$ and $K''_{36} = 9.4$; (—) (middle) calculated for $K''_{13} = 673.4$ and $K''_{36} = 5.2$; (···) calculated for $K''_{13} = 327$ and $K''_{36} = 5.4$; (-·-) calculated for $K''_{13} = 167$ and $K''_{36} = 6.3$; (—) (lower) calculated for $K''_{13} = 96$ and $K''_{36} = 7.3$.

Yphantis (1964), and zero concentration at the meniscus was achieved. An automatic analysis of data from the photoelectric scanner of the model E analytical ultracentrifuge has been developed (Kim et al., 1978). The concentration in OD units at different position inside the cell is obtained. The weight-average molecular weight at a position r, $\bar{M}_w(r)$, is

$$\bar{M}_w(r) = \frac{2RT}{(1 - \rho\bar{v})\omega^2} \frac{\mathrm{d} \ln c}{\mathrm{d}r^2} \quad (1)$$

where R is the gas constant, T is the absolute temperature, ρ is the density of the solution, $\bar{v}$ is the partial specific volume, ω is the rotor speed, r is the distance from the center of the rotor, and c is the OD units at 280 nm at the r position. A polynomial regression program was used on a PDP 1145 computer to obtain the best fit of ln c as a function of r^2. The first derivative of ln c with respect to r^2 gives the value of d ln c/dr^2 at any point in the cell. According to eq 1, the weight-average molecular weight, $\bar{M}_w(r)$, was calculated and plotted against the concentration in OD units at 280 nm. By extrapolation of the curve to zero concentration ($c = 0$), the molecular weight of the phycocyain monomer, the species favored by dilution, was found to be 35 000. This is in good agreement with the value obtained by other investigators (Kato et al., 1974; MacColl et al., 1980). The observed molecular weight of the monomer eliminated the possibility of the dissociation of the monomer to its subunits α and β at the lowest protein concentrations. The value of 35 000 for the monomer molecular weight was used throughout the entire calculation.

The proposed mechanism is

$$6\mathrm{M} \underset{}{\overset{K'_{13}}{\rightleftharpoons}} 2\mathrm{T} \overset{K'_{36}}{\rightleftharpoons} \mathrm{H} \quad (2)$$

$$K'_{13} = \frac{\bar{C}_T}{\bar{C}_M{}^3} \qquad K'_{36} = \frac{\bar{C}_H}{\bar{C}_T{}^2}$$

M represents monomer, T represents trimer, H represents hexamer, and K'_{13} and K'_{36} represent the equilibrium constants on a weight scale for each step. For the case when three species are present (monomer, trimer, and hexamer), the weight-average molecular weight $\bar{M}_w$ can be represented by

$$\bar{M}_w = (\bar{C}_M M_1 + \bar{C}_T M_3 + \bar{C}_H M_6)/C^0 \quad (3)$$

where M_1, M_3, and M_6 are the molecular weights of monomer, trimer, and hexamer, respectively, $\bar{C}_M$, $\bar{C}_T$, and $\bar{C}_H$ are the weight concentrations of the species at equilibrium, and C^0 is the total weight concentration of the solution. If we define χ such that $\bar{C}_M = \chi C^0$, then eq 3 can be rewritten as

$$C^0\bar{M}_w = (\chi C^0)M_1 + K'_{13}(\chi C^0)^3 M_3 + K'_{13}{}^2 K'_{36}(\chi C^0)^6 M_6 \quad (4)$$

Since $M_3 = 3M_1$, eq 4 can be simplified to

$$(\bar{M}_w/M_1\chi - 1)/(\chi C^0)^2 = 3K'_{13} + 6K'_{13}{}^2 K'_{36}(\chi C^0)^3 \quad (5)$$

Following Steiner's (1952) treatment, we can write

$$\ln \chi = \int_0^C [(\alpha^{-1} - 1)/C]\, \mathrm{d}C \quad (6)$$

where $\alpha = \bar{M}_w/M_1$. The values of χ as a function of C can be obtained by means of graphical integration. With these values, we can plot $(\bar{M}_w/M_1\chi^{-1})/(\chi C^0)^2$ as a function of $(\chi C^0)^3$. From the intercept and the slope, the value of $3K'_{13}$ and $6K'_{13}{}^2K'_{36}$, respectively, can be calculated. This plot is shown in Figure 6. By least squaring, we obtained the data values of $K''_{13} = 673.4$ and $K''_{36} = 5.2$ (in OD units). The extinction coefficient (ϵ) of phycocyanin at 280 nm in pH 4.8 acetate buffer was calculated to be 1.861 for a 1 mg/mL solution in a 1-cm cell. Assuming the same extinction coefficient for each species, we multiply K''_{13} and K''_{36} by $(1.2\epsilon)^2$ and 1.2ϵ, respectively, to obtain the equilibrium constants on a weight scale (K'_{13} and K'_{36}). The values are $K'_{13} = 3.4 \times 10^3$ (mL/mg)^2 and $K'_{36} = 11.6$ mL/mg. On a molar scale the equilibrium constants, K_{13} and K_{36}, are equal to $K'_{13}M_1{}^2/3$ and

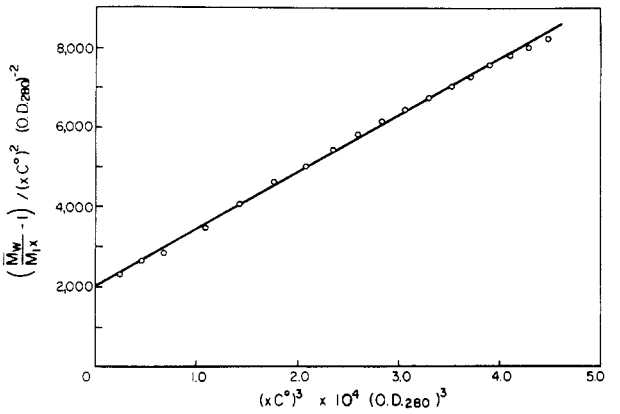

FIGURE 6: Plot of $\bar{M}_w/M_1\chi - 1)/(\chi C^0)^2$ vs. $(\chi C^0)^3$ values for C-phycocyanin at pH 4.8.

Table I: Equilibrium Constants and Gibbs Free Energies[a] at $T = 294.2$ K

parameters	weight scale	molar scale
K_{13}[b]	3.4×10^3	1.4×10^{12}
K_{36}[b]	11.6	6.1×10^5
K_{16}[b]	1.3×10^8	1.1×10^{30}
ΔG°_{13}		−16.3
ΔG°_{36}		−7.76
ΔG°_{16}		−40.3

[a] The unit for Gibbs free energy is kcal/mol. [b] The units are as follows: (mL/mg)^2 for K_{13}, mL/mg for K_{36}, (mL/mg)^5 for K_{16} on the weight scale and (L/mol)^2, L/mol, and (L/mol)^5, respectively, on the molar scale.

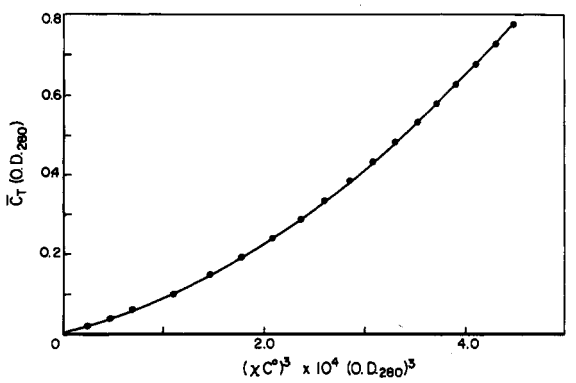

FIGURE 7: Plot of $\bar{C}_T$ values vs. $(\chi C^0)^3$ for monomer–trimer reaction.

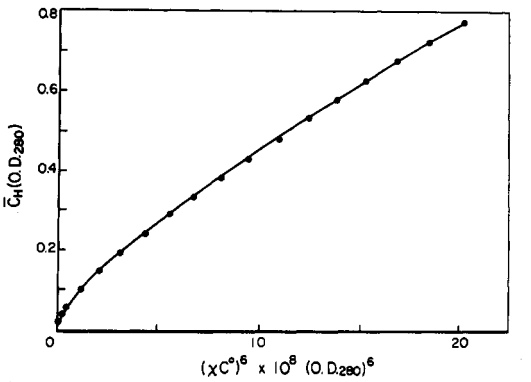

FIGURE 8: Plot of $\bar{C}_H$ values versus $(\chi C^0)^6$ for monomer–hexamer reaction.

K'_{36} $3M_1/2$, respectively, and are listed in Table I. Substituting the values of K'_{13} and K'_{36} into the equation

$$C^0 = \bar{C}_M + \bar{C}_T + \bar{C}_H = \bar{C}_M + K'_{13}\bar{C}_M{}^3 + K'_{13}{}^2K'_{36}\bar{C}_M{}^6 \quad (7)$$

We can estimate the concentrations of each species at a given C^0. The values of $\bar{M}_w$ as a function of C were calculated with eq 3. This calculated curve is given in Figure 5. In view of the experimental data, it is reasonable to conclude that the calculated curve fits the data fairly well. From the above analysis, it can be concluded that the self-association of phycocyanin leads to the presence of monomers, trimers, and hexamers in the mixture under these experimental conditions.

The analysis used to arrive at the proposed monomer–trimer–hexamer model and the specific equilibrium constants was tested in two ways. So that the sensitivity of the fitting procedure to the choice of reaction model could be tested, the following was considered. The equation for the association of n monomers into a n-mer is

$$n \text{ monomers} \rightleftharpoons n\text{-mer} \quad (8)$$

$$K'_n = \bar{C}_n/\bar{C}_M{}^n$$

where $\bar{C}_n$ and $\bar{C}_M$ are the weight concentration of the n-mer and the monomer, respectively, at equilibrium. Then K'_n is obtained from the slope of a plot of $\bar{C}_n$ vs. $\bar{C}_M{}^n$. From eq 6 the monomer and n-mer concentrations are calculated and their values are equal to χC^0 and $(1-\chi)C^0$, respectively. First considering the case of trimerization ($n = 3$), the values of $(1-\chi)C^0$ ($\bar{C}_T$) were plotted against the values of $(\chi C^0)^3$. The plot (Figure 7) showed a upward curvature. This fact, along with the observation that the experimentally determined weight-average molecular weights at high concentrations are greater than that of trimer, suggests that polymerization proceeded beyond trimerization. Similarly, the case of hexamerization ($n = 6$) was considered next. The plot of $(1-\chi)C^0$ ($=\bar{C}_H$) showed a downward curvature at lower concentrations (Figure 8). In view of the inability of the data to fit these two mechanisms for the association of phycocyanin, it may be postulated that the equilibrium mixture contains all the three species, monomers, trimers, and hexamers.

Next, the uniqueness of K''_{13} and K''_{36} was tested by employing several sets of these constants and calculating the weight-average molecular weight as a function of total protein concentration. The resulting data are given in Figure 5. Clearly, the curve resulting from the values of $K''_{13} = 673.4$ and $K''_{36} = 5.2$ is the best fit for monomer–trimer–hexamer reaction.

Discussion

The Spinco Model E ultracentrifuge equipped with the monochromator and photoelectric scanning systems has enabled us to demonstrate unequivocally the species present in phycocyanin solutions at low protein concentrations. In these experiments we have observed that the dissociation of the 19 S aggregate by pH treatment (pH 3.9) is irreversible. It is still not clear what is responsible for this irreversible reaction. For the protein solutions of preparation III, no definite plateau region was found between the 3 S and 11 S boundaries. This result suggested the possibility of the presence of the trimer species. The more accurate band-forming sedimentation velocity experiment on the Model E analytical ultracentrifuge was used in order to see whether the trimer is truly present. The results from band sedimentation velocity (Figure 4) strongly support the simultaneous presence of monomers, trimers, and hexamers.

It is desirable to examine the possibility for the other mechanisms by careful analysis of sedimentation equilibrium data. After removal of the 19 S aggregate by low pH treatment, there is a possibility of at most three species being present in a phycocyanin solution: monomers, trimers, and hexamers. Four possible mechanisms can be deduced as follows: (1) monomer $\rightleftharpoons$ trimer; (2) trimer $\rightleftharpoons$ hexamer; (3) monomer $\rightleftharpoons$ hexamer; (4) monomer $\rightleftharpoons$ trimer $\rightleftharpoons$ hexamer. From molecular weight measurements alone, we have carefully examined each mechanism for preparation III. For mechanism 1, if only monomers and trimers are present in the equilibrium system, then the weight-average molecular weight, $\bar{M}_w$, will have a maximum value of 105 000. It can be seen from Figure 5 that the trimerization hypothesis does not adequately explain the experimental data at high concentration. Similarly, the case of the trimer $\rightleftharpoons$ hexamer equilibrium system was considered next. As is to be expected, the minimum value of $\bar{M}_w$ will be 105 000, and this mechanism fails to fit the data at low concentration. For the monomer $\rightleftharpoons$ hexamer equilibrium system, the weight-average molecular weight, $\bar{M}_w$, can be represented as

$$\bar{M}_w = (\bar{C}_M M_1 + \bar{C}_H M_6)/C^0 \quad (9)$$

Since $\bar{C}_H = C^0 - \bar{C}_M$ and $M_6 = 6M_1$, it can be shown that

$$(\bar{M}_w - M_1)/(6M_1 - \bar{M}_w)^6 = K_{16}(C^0)^5/(5M_1)^5 \quad (10)$$

and

$$\ln [(\bar{M}_w - M_1)/(6M_1 - \bar{M}_w)^6] = \ln [K_{16}/(5M_1)^5] + \ln (C^0)^5$$

where K_{16} is the equilibrium constant. Figure 9 shows the plot of $\ln [(M_w - M_1)/(6M_1 - \bar{M}_w)^6]$ vs. $\ln (C^0)^5$. Equation 10 predicts that a value of the slope equal to one in the plot. A value of 0.42 for the slope was obtained from the plot. This is a straightforward method consistent with a mechanism which contains only two species. The only assumption is the

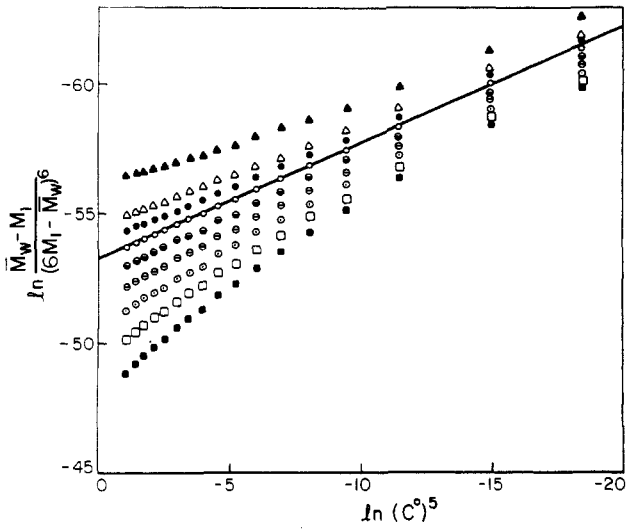

FIGURE 9: Plot of ln $[\bar{M}_w - M_1)/(6M_1 - \bar{M}_w)^6]$ vs. ln $(C^0)^5$ for C-phycocyanin at pH 4.8: (■) M_1 = 30000; (□) 31000; (⊙) 32000; (⊖) 33000; (⊜) 34000; (○) 35000; (●) 36000; (△) 37000; (▲) 40000.

molecular weight of the monomer. Different values of monomer molecular weight from 30000 to 40000 were used, and the various plots are shown in Figure 9. None of monomer molecular weight values can provide both the straight line fit and the right value of the slope in the plot. The only remaining mechanism is the coexistence of monomers, trimers, and hexamers, and the fitting of this equilibrium system has already been described under Results and Calculations. We have attempted to apply the same type of anlaysis for the mechanism outlined above to the sedimentation equilibrium data reported by Saito and co-workers (Saito et al., (1974, 1978; Iso et al., 1977). The test for the monomer–hexamer equilibrium using 9 and 10 and the plot in Figure 9 for their data does not result in a slope of 1.0 but instead one of about 1.5 with a large degree of scatter. In addition, an attempt similar to that in Figure 5 to fit experimental data to a calculated molecular weight as a function of concentration using their data and equilibrium constant results in a rather poor fit with large scatter. This would seem to point up the necessity of obtaining corraborating information for the presence or absence of species other than the monomer and hexamer and explicit calculations for alternative mechanisms other than the one that seems most attractive.

The Gibbs free energy change $\Delta G°$ accompaning the association–dissociation of phycocyanin at pH 4.8 can be calculated by using the relations

$$\Delta G°_{13} = -RT \ln K_{13}$$
$$\Delta G°_{36} = -RT \ln K_{36} \quad (11)$$

The $\Delta G°_{13}$ and $\Delta G°_{36}$ (T = 294.2 K) are calculated to be −16.3 and −7.76 kcal/mol, respectively. Iso et al. (1977) have studied the monomer ⇌ trimer reaction of phycocyanin from red alga, *Porphyra tenera*, in a phosphate buffer solution of pH 6.8 and an ionic strength of 0.1. The values of (2.0–4.0) × 10^{10} (L/mol)^2 and −13.1 to −14.4 kcal/mol were calculated to be the equilibrium constant (K_{13}) and the free energy change ($\Delta G°_{13}$). Chen & Berns (1977) have reported that the free energy change, ΔG, for the trimer ⇌ hexamer reaction is −5.6 kcal/mol in pH 6.0 phosphate buffer at 25 °C. Scott & Berns (1965) also indicated that under the same condition, the equilibrium constant (K_{36}) and the free energy change ($\Delta G°_{36}$) are equal to 4 × 10^4 L/mol and −6 kcal/mol, respectively. Their values are derived from the sedimentation velocity studies in which the real equilibrium is not achieved. For calculation of the standard free energy of the monomer ⇌ hexamer equilibrium system, the reaction

$$3 \text{ monomers} \rightleftharpoons \text{trimer} \quad (12)$$
$$\Delta G°_{13} = -16.3 \text{ kcal/mol}$$

is multiplied by 2 and added to the reaction

$$2 \text{ trimers} \rightleftharpoons \text{hexamer} \quad (13)$$
$$\Delta G°_{36} = -7.76 \text{ kcal/mol}$$

giving the reaction

$$6 \text{ monomers} \rightleftharpoons \text{hexamer} \quad (14)$$
$$\Delta G°_{16} = -40.3 \text{ kcal/mol}$$

This implies that the $\Delta G°_{16}$ value for the hexamer unit is evaluated to be −40.3 kcal/mol. This result is not so different from the value of $\Delta G°$ reported by Saito et al. (1978). By definition, the association constant of the monomer ⇌ hexamer reaction (K_{16}) is equal to $K_{13}^2 K_{36}$ and calculated to be 1.1 × 10^{30} (L/mol)^5. The equilibrium constants and corresponding free energies for each step are listed in Table I. MacColl et al. (1971b) have reported that the association constant for the monomer to hexamer is in the order of 10^{30} in pH 6.0 phosphate buffer. From the negative values of $\Delta G°_{13}$ and $\Delta G°_{36}$ for the phycocyanin aggregation obtained in this study, it is clear that the self-association of C-phycocyanin is spontaneous at pH 4.8.

It is important to note that different distributions of species are obtained by different methods of preparations and therefore the history of sample purification and buffer exposure is extremely important as mentioned under Results and Calculations. Extrapolation of conclusions concerning equilibrium constants and distribution of aggregates should take into account potential differences resulting from different sample preparation conditions and general sample history.

This work provides good characterization for the conditions to be employed with kinetic studies in stopped-flow experiments. We can pursue the monomer ⇌ hexamer reaction under preparation II and the monomer ⇌ trimer ⇌ hexamer reaction under preparation III. It is very important to understand the formation steps of individual biliprotein aggregates. Subsequent experiments to investigate higher aggregate formation in C-phycocyanin and with other biliproteins that should be of relevance in phycobilisome formation will be pursued by using kinetic and equilibrium experiments under carefully selected conditions.

References

Berns, D. S., & Morgenstern, A. (1966) *Biochemistry 5*, 2985–2990.

Chen, C. H., & Berns, D. S. (1977) *J. Phys. Chem. 81*, 125–129.

Gantt, E. (1975) *BioScience 25*, 781–788.

Gantt, E., Lipschultz, C. A., & Zilinskas, B. A. (1976) *Biochim. Biophys. Acta 430*, 375–388.

Glazer, A. N. (1976) *Photochem. Photobiol. Rev. 1*, 71–115.

Iso, N., Mizuno, H., Saito, T., Nitta, N., & Yoshizaki, K. (1977) *Bull. Chem. Soc. Jpn. 50*, 2892–2895.

Kao, O., Berns, D. S., & MacColl, R. (1971) *Eur. J. Biochem. 19*, 595–599.

Kato, M., Lee, W. I., Eichinger, B. E., & Schurr, J. M. (1974) *Biopolymers 13*, 2293–2304.

Kim, C. S., Moross, G., Moyer, W., Kaufman, G., MacColl, R., & Edwards, M. R. (1978) *Anal. Biochem. 86*, 371–377.

Lee, J. J., & Berns, D. S. (1968) *Biochem. J. 110*, 465–470.
MacColl, R., Berns, D. S., & Koven, N. L. (1971a) *Arch. Biochem. Biophys. 146*, 477–482.
MacColl, R., Lee, J. J., & Berns, D. S. (1971b) *Biochem. J. 122*, 421–426.
MacColl, R., Csatorday, K., Berns, D. S., & Traeger, E. (1980) *Biochemistry 19*, 2817–2820.
Saito, T., Iso, M. N., & Mizuno, H. (1974) *Bull. Chem. Soc. Jpn. 47*, 1375–1379.
Saito, T., Iso, N., Mizuno, H., & Kitamura, I. (1978) *Bull. Chem. Soc. Jpn. 51*, 3471–3474.
Scott, E. (1965) Ph.D. Thesis, Albany Medical College, Union University, Albany, NY.
Scott, E., & Berns, D. S. (1965) *Biochemistry 4*, 2597–2606.
Steiner, R. F. (1952) *Arch. Biochem. Biophys. 39*, 333–354.
Viograd, J., Bruner, R., Kent, R., & Weigle, J. (1963) *Proc. Natl. Acad. Sci. U.S.A. 49*, 902–910.
Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.

Fluorescent Probe of Ribonuclease A Conformation†

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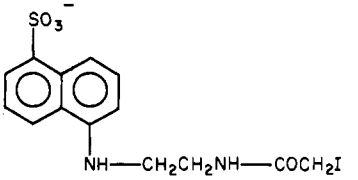
ABSTRACT: The reaction of ribonuclease (RNase) with *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid (1,5-IAENS) yields a derivative in which one fluorescent group is covalently attached to the protein. Several arguments suggest that the chemical modification has occurred at the enzyme active site: (i) 1,5-IAENS should have the same specificity as iodoacetamide, i.e., carboxymethylate one histidine of the active site; (ii) the derivatized protein is enzymatically inactive; (iii) in the native state of the protein, the fluorescent group is (almost) completely protected from the aqueous solvent; (iv) this group has no motions other than those of the protein. The fluorescence properties of the derivatized RNase change markedly upon unfolding induced by guanidine hydrochloride (Gdn·HCl), as seen from fluorescence intensity, maximum emission wavelength, and polarization measurements. Upon Gdn·HCl-induced unfolding, the fluorescent group is transferred from a nonpolar to a highly polar environment. Dynamic fluorescence measurements show also that unfolding results in a markedly increased mobility of the fluorescent label with respect to its proteic environment. These results are compared to those of Young & Potts (1963) [Young, D. M., & Potts, J. T. (1963) *J. Biol. Chem. 238*, 1995–2002], who studied the fluorescence properties of a surface-labeled derivative of RNase.

The use of reporter groups has proved to be a useful technique for studying conformational changes in proteins. The changes observed in a given property of the reporter group(s) are assumed to reflect changes in its environment as provided by the protein. Such reporter groups can be either built in the protein structure (e.g., aromatic residues, prosthetic groups, etc.) or introduced into the protein by a suitable chemical modification. A most desirable situation is when a single reporter group exists, because the origin of the observed changes can be unambiguously assigned to a defined region of the protein. Among all the conformational changes which can take place within a polypeptide chain, the major one, at least in amplitude, is its folding, i.e., the process by which this chain acquires its unique, compact, native structure. Ribonuclease (RNase)[1] is one of the small globular proteins the folding process of which has been, and still is, extensively studied, and the present paper shows that a fluorescent reporter group can be introduced into RNase and that it can be used to monitor folding of the protein.

The reason for choosing fluorescence as the observed parameter is that different information can be obtained depending on the type of measurement (Stryer, 1968). Fluorescence intensity, maximum emission wavelength, and lifetime are influenced by the properties of the close environment of the probe, such as polarity, solvation, etc. Fluorescence polarization and anisotropy decay depend on the mobility of the probe and can report on the relative motions of the probe and its environment. Therefore, a fluorescent probe can be characterized both by its static and by its dynamic behavior in different conformational states of the protein. RNase has no tryptophan; its intrinsic fluorescence is only due to its six tyrosines. Tyrosine fluorescence has been used to measure RNase folding, but only as an overall parameter, through fluorescence intensity (Schmid, 1981).

The strategy for introducing a covalent fluorescent probe into RNase rests on the particular reactivity of the active site toward halo acids and their amide derivatives. At slightly acidic pH, these reagents modify almost specifically one histidine in native RNase, either His-12 or His-119, but not both of them (Richards & Wyckoff, 1971). RNase was therefore reacted with *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid (1,5-IAENS), a reagent which combines a fluorescent group similar to dansyl and a reactive end resembling iodoacetamide, separated by free-rotating bonds (Hudson & Weber, 1973):



The reaction of 1,5-IAENS with RNase yields a chemical derivative, AENS-RNase, which has one fluorescent group

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[1] Abbreviations used: RNase, bovine pancreatic ribonuclease; 1,5-IAENS, *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid; AENS, (acetamidoamino)ethyl-5-naphthylamine-1-sulfonic acid; Gdn·HCl, guanidine hydrochloride; 2′,3′-CMP, cytidine cyclic 2′,3′-phosphate; 2′-CMP, cytidine 2′-phosphate.

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