Protein Aggregation in C-Phycocyanin

STUDIES AT VERY LOW CONCENTRATIONS WITH THE PHOTOELECTRIC SCANNER OF THE ULTRACENTRIFUGE

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Solutions of C-phycocyanin of very low concentrations were examined by sedimentation-velocity studies in the Spinco model E ultracentrifuge equipped with a photoelectric scanning system and a monochromator. At sufficiently low concentrations complete disaggregation from the hexamer to the monomer was observed. The equilibrium constant of monomer to hexamer was estimated to be approx. 10^{30} . For studies of aggregation over the complete range of concentration, C-phycocyanins from *Phormidium luridum* and *Lyngbya* sp. were used. Sedimentation-velocity studies at high concentration with schlieren optics are reported for C-phycocyanins from *Anabaena variabilis* and *Lyngbya* sp. The pH-dependence of aggregation and the temperature-dependence of trimer-hexamer equilibrium for phycocyanins from these algae were found to be similar to those of other C-phycocyanins. The principal feature of the pH-dependence is the dominance of hexamers at the isoelectric point. Increasing temperature increased the amount of hexamer and decreased the amount of trimer.

Several studies on the aggregation properties of C-phycocyanin have been reported from this laboratory (Scott & Bernis, 1965; Bernis & Morgenstern, 1966). The relative amount of the several aggregates under specified conditions is insensitive to the total phycocyanin concentration over the range 4–40 mg/ml (Scott & Bernis, 1965). Even at concentrations as low as 0.2 mg/ml substantial amounts of large aggregates are present (Bernis & Morgenstern, 1966). Incomplete disaggregation into monomer at low concentrations presented the question whether equilibrium exists between the various aggregates. Reversible temperature and pH effects argue in favour of equilibrium between the trimer and hexamer species. The present paper presents new evidence to support the concept of an equilibrium among the aggregates. These experiments demonstrate that a concentration-dependence exists and complete disaggregation is obtained when sufficiently low concentrations are reached. The previously observed lack of disaggregation at the lowest concentration reached by Bernis & Morgenstern (1966) resulted from a very large association constant and is analogous to micelle formation. Results comparing aggregation of phycocyanins from several different algae are presented. Four phycocyanin species important in this study are monomer, trimer, hexamer and dodecamer, and are designated as 3S, 6S, 11S and 19S respectively.

EXPERIMENTAL

Treatment of C-phycocyanin. Phycocyanin was isolated from laboratory cultures of the blue-green algae *Phormidium luridum*, *Anabaena variabilis* and *Lyngbya* sp. Harvests of the last two algae were kindly supplied by Dr H. W. Siegelman of Brookhaven National Laboratory, Upton, N.Y., U.S.A. All algal cultures were obtained from the Indiana University culture collection. The protein was extracted and purified at pH 6.0 by methods described by Scott & Bernis (1965). The E_{620}/E_{280} ratio for all protein solutions was 4.0 or greater in sodium phosphate buffer, pH 6.0 and I 0.1. All proteins were purified so that no species was observed to sediment faster than 19S. Phycocyanins from *Phormidium luridum* and *Lyngbya* sp. were employed for experiments performed at concentrations below 0.1 mg/ml.

Protein concentrations were determined from the extinction at 620 nm with $E_{1\%}^{1\text{cm}}$ 6.0. Protein solutions were also prepared by weighing freeze-dried protein and correcting for the calculated amount of residual water. The two methods produced consistent results. Extinction readings were obtained on either a Gilford model 2400 or a Cary 14 spectrophotometer. A 0.1 E slide wire was used on the Cary 14 instrument for very dilute solutions.

Ultracentrifuge studies with absorption optics. Sedimentation-velocity studies were performed with a Spinco model E ultracentrifuge equipped with absorption optics. This absorption system included a high-intensity xenon light-source, a monochromator, a photoelectric scanner and a multiplexer. Solutions were scanned in the centrifuge at 278, 423, 584 or 620 nm. The different extinction

coefficients at these wavelengths allowed investigation of a wide range of concentrations. The most dilute solutions were scanned at 584 or 620 nm with an expanded recorder scale. Most of the phycocyanin solutions were scanned in a cell with a 12 mm double-sector centrepiece and sapphire windows at a speed of 52640 rev./min in an AN-F rotor. In a few cases a 30 mm double-sector centrepiece was employed at 50760 rev./min in an AN-E rotor.

The proportion of each component was determined as the relative extinction associated with each boundary. The assumption that all phycocyanin species have the same extinction coefficient introduced minor error in the quantitative aspect of the results.

Ultracentrifuge studies with schlieren optics. Phycocyanin solutions at concentrations above 3 mg/ml were studied by sedimentation velocity in the Spinco model E ultracentrifuge with schlieren optics. For these experiments an AN-D rotor was used at 59780 rev./min in conjunction with a Corning no. 5031 filter and Kodak type 1-N spectroscopic plates.

Ultracentrifuge studies with band-forming centrepiece. The sedimentation-velocity studies of phycocyanins were also performed with a double-sector analytical band-forming centrepiece (Vinograd, Bruner, Kent & Weigle, 1963) together with the photoelectric scanning system in the Spinco model E ultracentrifuge. In these experiments 10 μ l of phycocyanin solution, containing between 0.12 and 1.0 mg/ml, was layered on to a bulk solution of 50 or 99.9% D₂O in sodium phosphate buffer, pH 6 and *I* 0.1. Band sedimentation-velocity centrifugation results in physical separation of the bands. Studies with the usual boundary sedimentation-velocity method established that a 50% D₂O solution did not change the relative amounts of the aggregates from those found in an H₂O solution at the same pH and ionic strength. The results obtained with 99.9% D₂O solutions showed an increase in the 11S species and a corresponding decrease in the 6S species compared with the results with a 50% D₂O solution. Sedimentation coefficients determined in 99.9% D₂O were corrected to correspond to water at 20°C as described by Lee & Bernis (1968b).

Buffers and chemicals. All buffers had *I* 0.1. Sodium phosphate buffers were used at pH 6.0, 7.0 and 8.0, and sodium acetate-acetic acid buffers between pH 5.4 and 3.9. D₂O (99.90%) was from Bio-Rad Laboratories, Richmond, Calif., U.S.A.

RESULTS

Physical properties of C-phycocyanin aggregation. The phycocyanins purified for this study had similar aggregation properties regardless of the algal source. Aggregates with similar sedimentation coefficients and the same pH- and temperature-dependence were obtained for *Anabaena variabilis* and *Lyngbya* sp. Fig. 1 presents the pH-dependence of the proportions of 3S, 6S, 11S and 19S species. Similar pH-dependence for *Lyngbya* sp. and *Anabaena variabilis* was observed. Increasing the temperature resulted in an increase in the 11S species and a decrease in the 6S species for phycocyanins from all algae examined. Phycocyanin from *Lyngbya* sp. at 13 mg/ml and pH 6.0 showed an increase in the 11S species from 38 to 65% and a decrease in the 6S species from 56 to 25% when the temperature was raised from 3.1°C to 25°C. The reversibility between the 11S and 6S species was also demonstrated for these various algae by changing pH. When the pH of the solution was changed from pH 8 to 6 by dialysis, the proportions of the 11S and 6S species reverted to the values normally obtained at pH 6. The amount of the 19S species, however, remained unchanged from its value at pH 8.

Concentration-dependence of the aggregates. In the phycocyanin solutions at pH 6.0 used in the concentration studies, three boundaries (19S, 11S and 6S) were observed in schlieren sedimentation-

Fig. 1. pH-dependence of the phycocyanin aggregates in the pH range 4.7–8.0. Percentages are calculated from the areas under schlieren peaks. The phycocyanins were from: (a) *Anabaena variabilis*; (b) *Lyngbya* sp.; (c) *Plectonema calothricoides* (values taken from Scott & Bernis, 1965). Phycocyanin aggregates; Δ , 3S; $\blacktriangle$, 6S; $\circ$, 11S; $\bullet$, 19S.

Fig. 2. Typical schlieren patterns for solutions of phycocyanins at pH 6.0. All sedimentation is from left to right at 59780 rev./min in the Spinco model E ultracentrifuge at 24 min after full speed was reached. The phycocyanins were from: (a) *Lyngbya* sp.; (b) *Phormidium luridum*.

Fig. 3. Sedimentation patterns as a function of time for a solution of phycocyanin (0.24 mg/ml) from *Lyngbya* sp. with the photoelectric scanning system on the Spinco model E ultracentrifuge. Light of wavelength 620 nm was used for all scans. M, Location of meniscus; CB, location of the cell bottom; RH, location of outer reference hole; CT, location of cell top. The speed was 52640 rev./min at 25°C. The times after full speed was reached were: (a) 10 min; (b) 27 min; (c) 90 min.

velocity patterns. In some cases a very small boundary was observed that corresponded to a 3S species. At over 3 mg/ml the relative amounts of the aggregates remained essentially constant. The

Fig. 4. Plot of relative amounts of monomer (3S), trimer (6S), hexamer (11S) and dodecamer (19S) as a function of total concentration of phycocyanin from *Lyngbya* sp. Data are from sedimentation-velocity experiments with absorption (620 and 278 nm) and schlieren optics at 25°C. Phycocyanin aggregates: Δ , 3S; $\blacktriangle$, 6S; $\circ$, 11S; $\bullet$, 19S. The disaggregation of phycocyanin from *Phormidium luridum* produced a similar plot of this type with absorption optics (584, 423 and 278 nm) and schlieren optics.

apparent concentration-independence was observed for phycocyanins from all algal sources examined. Typical sedimentation-velocity patterns for the phycocyanin components from the blue-green algae used in the concentration-dependence study are shown in Fig. 2.

Four species were observed when concentrations lower than those examined by schlieren optics were investigated with absorption optics (Fig. 3). However, below 0.1 mg/ml the 6S species began to disappear rapidly and the 3S monomer became the predominant species. At the lowest concentrations used for sedimentation-velocity studies with absorption optics the higher aggregates disappeared and eventually only the 3S species remained. A plot of relative proportion against total protein concentration (Fig. 4) for the entire concentration range studied by both schlieren and absorption optics demonstrated that the 11S species was the last to disaggregate entirely to the 3S species. Phycocyanins from both *Phormidium luridum* and *Lyngbya* sp. disaggregated in a similar manner. In both cases and at similar concentrations the 11S species was the last to disappear and the 6S species was the first. The greatest difficulty in analysing the results was in the determination of the exact plateau point between the broad 3S and 6S boundaries. Thus in the concentration region where both 6S and 3S boundaries exist, a large amount of uncertainty must be associated with the proportions calculated for these species. At the lowest concentrations examined where the 6S species had completely disaggregated, definite plateau regions were found between the 11S and 19S and the 3S and 11S boundaries (Fig. 5). The low-concentration results with phycocyanin from *Lyngbya* sp. in Fig. 4 were obtained at 620 nm; similar results were obtained

Fig. 5. Sedimentation patterns obtained for phycocyanin from *Phormidium luridum* with a photoelectric scanning system on the Spinco model E ultracentrifuge. Light with a wavelength of 584nm was used for all scans. Abbreviations are as for Fig. 3. The speed was 52 640 rev./min at 23°C for all samples. Phycocyanin concentrations: (a) 0.008 mg/ml, 32 min, single boundary observed; (b) 0.028 mg/ml, 36 min; (c) 0.056 mg/ml, 38 min.

Fig. 6. Plot of monomer concentration of phycocyanin from *Lyngbya* sp. as a function of total phycocyanin concentration. —, Calculated curve for $6M \rightleftharpoons M_6$, $M = 3 \times 10^4$, and $K = 10^{30}$; ○ and ----, experimental points obtained for the monomer concentration, calculated from sedimentation-velocity experiments on the Spinco model E ultracentrifuge.

at 584nm with phycocyanin from *Phormidium luridum*. The same trends observed at both 620nm and 584nm indicate that the disaggregation behaviour probably is not an artifact of the scanning wavelength, but is a true indication of the system's behaviour. A plot of actual monomer concentration against total phycocyanin concentration is presented in Fig. 6. This presentation is important in comparing the results with a calculated curve for the monomer-hexamer equilibrium.

Fig. 7. Sedimentation pattern for a solution of phycocyanin (0.20 mg/ml) from *Anabaena variabilis* with the photoelectric scanner on the Spinco model E ultracentrifuge and band-forming centrepiece. Scans were made at 620nm at 52 640 rev./min and 25°C. The time after full speed was reached was 37 min. Abbreviations are as for Fig. 3.

Fig. 8. Plot of the sedimentation coefficient as a function of concentration for the band-forming experiments on phycocyanin from *Lyngbya* sp. The concentrations are for the initial 10 μ l before the layering. All sedimentation coefficients were first corrected to correspond to 20°C and H_2O . ▲ and △, samples layered on 99.9% D_2O ; ● and ○, samples layered over 50% D_2O .

With decreasing protein concentration, the conversion of hexamer into monomer was also observed at pH 4.6. At this pH, 23°C and 1.0 l, a 0.13 mg/ml solution of phycocyanin from *Phormidium luridum* had 25% of the 11S species and 75% of the 3S species. At 0.058 mg/ml, the 11S species was 11% and the 3S was 89%. Only 3S and 11S boundaries were observed in these solutions. The absence of any 6S species demonstrates that the 11S species dissociates directly to 3S species.

Results from band-forming centrepieces. The boundaries studied in these sedimentation-velocity experiments are undoubtedly due to very complex mixtures of species. Therefore sedimentation-velocity studies with absorption optics at 620nm

were performed with band-forming centrepieces. The separated bands represented distinct uncontaminated species. When a phycocyanin solution was placed on a 50% D₂O column at pH 6, three bands were seen (Fig. 7). The $s_{20,w}^0$ values for the two fastest-sedimenting bands were 16.8S and 10.7S (Fig. 8). The slowest band was probably a combination of 6S and 3S species since, after a relatively long sedimentation time, it partially split into two bands.

DISCUSSION

The monochromator and photoelectric scanning systems in the Spinco model E ultracentrifuge have enabled us to demonstrate unequivocally that the species in phycocyanin solutions sedimenting at 3S, 6S, 11S and 19S each absorb in the 620nm region. This result suggests that species observed by sedimentation-velocity experiments with absorption optics are phycocyanin monomer and aggregates. The results from band-sedimentation experiments strongly support this conclusion. The band experiments resolve uncontaminated 11S and 19S peaks that absorb at 620nm (Fig. 7). Likewise, since absorption and schlieren optics give equivalent results with respect to the number of species and their sedimentation coefficients, all the species observed by schlieren optics are probably phycocyanin. This correlation confirms the earlier assignment of 6S, 11S and 19S for phycocyanin aggregates. In addition, the same number of boundaries with the same sedimentation coefficients was found at all the wavelengths investigated with absorption optics.

The several phycocyanins used in this study had similar aggregation properties as judged from analysis of sedimentation-velocity patterns with schlieren optics. Phycocyanin from *Plectonema calothricoides* (Scott & Berns, 1965) also exhibited aggregates with sedimentation coefficients and temperature- and pH-dependence identical with those shown here. The salient features of this behaviour (Fig. 1) are the predominance of the 11S species at pH values near the isoelectric point (pH 4.7) and the increase in the 6S species when the pH is raised above the isoelectric point. When the temperature is increased, 6S species is converted into 11S species. These results have been interpreted by Scott & Berns (1965) as an indication that the interactions forming the 11S species are hydrophobic. Phycocyanins from *Anacystis nidulans* (Neufeld & Riggs, 1969) and from the thermophile *Synechococcus lividus* (Berns & Scott, 1966) also show a similar pH-dependence.

The most obvious difference in the various reports on the aggregation of phycocyanin is in the number of aggregates. This difference seems to be

easily explained as a consequence of purification techniques. Phycocyanin purified from *Phormidium luridum* in previous experiments in this laboratory (Lee & Berns, 1968a) showed an aggregate even larger than 19S. This higher aggregate resulted from fast and gentle purification and was also observed in crude extracts. In contrast, phycocyanin purified from *Anabaena variabilis* by Craig & Carr (1968) showed 11S and 6S species, but no 19S species, at pH 6.8. Fig. 1 shows that phycocyanin from *Anabaena variabilis* in this research has about 36% of 19S species at pH 6.0 and 16% at pH 7.0. The lack of 19S species in the former case can be explained since Craig & Carr (1968) purified their material at pH 6.8 and utilized gel filtration in their procedures.

The importance of the higher aggregates to the functions of algal cells and the major roles that the 11S species assumes *in vivo* have been discussed (Scott & Berns, 1965; Berns & Edwards, 1965; Lee & Berns, 1968a,b). The results of the disaggregation to monomer as a function of concentration lend credence to the concept that the 11S species is a vital ingredient in a step-by-step phycocyanin assembly in the cell. The hexamer (Berns & Edwards, 1965) was the last aggregate to dissociate into monomer units. The dissociation process occurred at very low concentrations and therefore resulted in a high association constant. Measurable amounts of hexamer were still obtained with *Lyngbya* sp. at about 0.008mg/ml. The equilibrium constant, K , for the interconversion: 6 monomer $\rightleftharpoons$ 1 hexamer, for phycocyanin from *Lyngbya* sp. was estimated at 25°C and pH 6.0, assuming a monomer molecular weight of 30000 (Kao & Berns, 1968; Berns & Kao, 1969) and an 11S molecular weight of 180000, to be approx. 10^{30} . This high equilibrium constant ensures a rapid increase in the amount of hexamer as the concentration is increased. These hexamers are thus readily available to undergo extensive polymerization to higher aggregates. Apparently the 6S species was not an essential step in the dissociation of the 11S species to the 3S species, since at very low concentrations this process occurred without measurable quantities of the 6S species being present.

In these experiments we have shown that the aggregates can be dissociated to monomer. The reverse reaction occurred when a solution at pH 3.9 that contained almost 100% of monomer was dialysed to pH 4.7. The resulting solution, when analysed by sedimentation velocity with schlieren optics, contained about 85% of hexamer and 15% of monomer. No 19S or 6S species was obtained.

The assignment of 19S and 11S species to regions shown in Fig. 3 is well substantiated by the band-sedimentation results (Fig. 7). However, as stated above, the lack of a clear plateau between the 6S

and 3S boundaries suggests that the calculated amounts of each are unreliable. In Fig. 6 the experimental points lie in good agreement with the calculated curve for: $6M \rightleftharpoons M_6$, mol.wt. of monomer $= 3 \times 10^4$, and $K = 10^{30}$. This good correlation suggests that the method used to discriminate between the 6S and 3S species is essentially accurate.

The effect of temperature (Scott & Bernes, 1965), D_2O (Bernes, Lee & Scott, 1968; Lee & Bernes, 1968b) and guanidinium salts (Bernes & Morgenstern, 1968) on the stability of the phycocyanin hexamer indicates that it is formed by hydrophobic interactions and Bernes & Morgenstern (1968) have compared the formation of hexamer with micelle formation. The very large value (10^{30}) for the association constant of the monomer-hexamer reaction and consequently the monomer concentration plot (Fig. 6) also bear similarity to the results obtained in the formation of micelles from detergent monomers.

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