Protein Aggregation in a Thermophilic Protein. Phycocyanin from *Synechococcus lividus**

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ABSTRACT: The aggregation of phycocyanin from a thermophilic Cyanophyta, *Synechococcus lividus*, has been studied as a function of pH by ultracentrifugation and immunodiffusion. The general behavior of the thermophilic protein is analogous to that observed with phycocyanin isolated from Cyanophyta grown at ~25°. When experiments are conducted at ~25° there is more 7 S or electrostatic trimer at lower pH values with the thermophilic protein than is observed with normal phycocyanins. As the temperature is raised to near 50°, the culturing temperature of *S. lividus*, the distribution of aggregating species becomes almost identical with that observed in the normal phycocyanins at ~25°. There are much less 7S and much greater amounts of 11S and 19S material. It is postulated that a greater number of charged and polar residues in the *S. lividus* protein account for the greater electrostatic interactions and hence a temperature near the 50° cultivation temperature is necessary to overcome this nonspecific interaction and to have formation of the postulated important 11S and 19S aggregates *in vivo* which are evidently not held together by nonspecific charge interactions. The observations are consistent with the previously suggested importance *in vivo* of the 11S and 19S aggregates and also present a mechanism by which aggregating proteins from thermophilic organisms may adapt to the proper aggregation equilibria at higher temperatures.

In previous studies of the reversible aggregation of the C-phycocyanin system (Scott and Berns, 1965; Berns and Edwards, 1965; Berns *et al.*, 1964), we have demonstrated the pH, ionic strength, and temperature dependence of the aggregation by examination of the ultracentrifuge patterns, intrinsic viscosity, spectra, and electron micrographs of C-phycocyanin isolated from the alga *Plectonema calothricoides*. We have postulated that the 11S sedimenting species that we propose to be the hexameric species is the important species *in vivo* and we have also suggested that the 19S sedimenting species is a dodecamer and is possibly important *in vivo*. The types of forces involved in the proposed monomer–trimer–hexamer–dodecamer equilibrium were also characterized. It, therefore, appears important to characterize the aggregation in phycocyanins from other algal sources to be assured of the general nature of the aggregation and the significance of our interpretation. C-phycocyanin from the alga *Phormidium luridum* has been investigated in detail and has been found to be almost identical in its properties with the phycocyanin extracted from *P. calothricoides*. This is important for consideration of the general behavior of this aggregating system. Both *P. calothricoides* and *Ph. luridum* are filamentous algae classified as Cyanophyta which grow in similar media and at similar temperatures, *ca.* 25–30°. The alga *Synechococcus lividus* is also classified as a Cyanophyta; however, it is a thermophile and normal laboratory culturing conditions require 55°. Using the observed temperature dependence of the aggregation of C-phycocyanin (Scott and Berns, 1965) from *P. calothricoides* as a basis, we postulated that when the thermophilic system is examined at 25° in the ultracentrifuge, the behavior should be similar to that for the normal phycocyanin system at *ca.* 5°. To obtain the comparable distribution of aggregates observed at 25° for the *P. calothricoides* system, we should, therefore, need to increase the temperature to *ca.* 45°. The results of our experiments confirm this postulated behavior.

Experimental Section

Materials and Methods. The initial cultures of *S. lividus* were kindly furnished by Dr. J. Middlebrook of Ling-Temco-Vought Research Center in Dallas, Texas, and Dr. D. L. Dyer of Northrup Space Laboratories, Hawthorne, Calif. The culture was maintained and grown at 55° in 1-l. batches on algae medium described by Allen and Arnon (1955). The cultures were autotrophically grown with Matheson biological atmosphere (5% CO_2 95% N) on a rotating shaker with fluorescent illumination. The method of cell lysing and collection of phycocyanin in the supernatant was similar to that previously described (Scott and Berns, 1965). Separation of the allophycocyanin from *S. lividus* required more than the usual ammonium sulfate fractionation. Several additional 35 and 40% ammonium sulfate cuts were necessary. It was possible

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to purify this protein by the ammonium sulfate fractionation method without using any of the calcium phosphate, ion-exchange column, or batch procedures. Comparison of salt fraction purified preparations with preparations subsequently treated with any of the several column procedures (Scott and Berns, 1965) demonstrated essentially no increase in purity by any of the established criteria (Scott and Berns, 1965), with the separation of little if any allophycocyanin. The use of the calcium phosphate procedures and ion-exchange procedures has been demonstrated in our laboratory[1] to be deleterious to the ability of the native protein to aggregate reversibly. The ratio of the 620-mμ absorption to the 280-mμ absorption was >4 for all preparations in these studies. All buffers used were identical with those in our previous work (Scott and Berns, 1965) with analytical reagents always employed.

Experiments were performed on a Spinco Model E ultracentrifuge, with a Philpot–Svenson cylindrical lens, diagonal bar system. Type 1N Kodak spectroscopic plates and a Corning No. 5031 filter were used because of the intense blue absorption of the protein. All centrifuge examinations were performed with a 60° bar angle and a rotor temperature indicator and control system when possible. The higher temperature tests were performed by preheating the rotor to about 50°. All distances were measured on a Nikon microcomparator. Relative areas were measured from enlarged tracings of the centrifuge patterns by counting squares on appropriate graph paper.

Electrophoresis. Cellulose acetate strip electrophoresis was simultaneously performed on samples of *P. calothricoides*, *S. lividus*, and *Ph. luridum* phycocyanin with sodium acetate buffers at pH 4.0, 4.7, and 5.0, all at an ionic strength of 0.02. Duplicate samples of each were examined and the voltage was maintained at 400 v for 2–3 hr using a Buchler power supply. Free solution electrophoresis of the *S. lividus* phycocyanin at the above-mentioned pH values was attempted but had to be abandoned because of the very low solubility of the protein.

Immunochemical Studies. Rabbits were inoculated with a suspension of equal volumes of phycocyanin and complete Freund's adjuvant. Each rabbit was injected with *ca.* 3 mg of protein; *ca.* 0.3 ml of suspension was injected into each toepad and 0.4 ml subcutaneously in the back of the neck. One month later the injections were repeated, but with 1 ml of suspension subcutaneously in the neck. After the animals rested for 1 week, 50 ml of blood was collected from each by cardiac puncture. First-course sera were used in all studies. Phycocyanin from *S. lividus*, *P. calothricoides*, and *Ph. luridum* were used as antigens as was phycocyanin from *P. calothricoides* treated with 1% sodium dodecyl sulfate. The Ouchterlony plates were set up as described by Kabat and Mayer (1961) and by Allison and Humphrey (1960). The latter method was used to evaluate diffusion coefficients as reported previously (Scott and Berns, 1965). Ouchterlony plates were set up with antisera to phycocyanins from *P. calothricoides*, *Ph. luridum*, or *S. lividus* diffusing *vs.* the homologous antigens and several heterologous antigens. Ouchterlony diffusion studies were carried out with 1.5% agar in physiological saline and also in appropriate buffers corresponding exactly to those used in the sedimentation studies. The plates were photographed after inspection under a long-wavelength ultraviolet lamp to ascertain that all precipitin lines exhibited the red fluorescence characteristic of phycocyanin.

TABLE I: pH Effects on Sedimentation.

pH[a]	*S. lividus* Phycocyanin s_{25}	*S. lividus* Phycocyanin Relative Area	*P. calothricoides* Phycocyanin[b] s_{25}	*P. calothricoides* Phycocyanin[b] Relative Area
			3.7[c]	6
5.0 acetate[d]	6.6[c]	10	6.3	10
	11.9[c]	64	12.1	75
	17.5[c]	25	18.9	9
6.0 phosphate[e]	5.7[c]	45	6.2	25
	11.4[c]	55	12.6	68
	15.0[c]	5	19.2	8
7.0 phosphate	5.7[c]	95	7.0	62
	~11[c]	~5	12.9	32
	~19[c]	Trace	20.8	7
7.0 cacodylic	5.7[c]	75	6.9[c]	27
	9.9[c]	25	10.2[c]	63
	~19[c]	Trace	18.6[c]	11
8.0 phosphate	5.6[c]	~100	6.5	65
			12.5	31
			20.1	5
9.0 carbonate	6.8[f]	100	5.2[f]	93
			18.9	7

[a] All of ionic strength 0.1. [b] From Scott and Berns (1965). [c] Sedimentation values for a single determination—not extrapolated. [d] The data for *S. lividus* are at pH 5.5, μ = 0.1, phosphate; schlieren sedimentation studies at pH 5.0, and pH 5.5 acetate with *S. lividus* indicated analogous data. However, the protein was only slightly soluble at pH 5.0 and 5.5 and sedimentation patterns could not be analyzed for per cent composition with any reliability. At pH 5.5 in phosphate, the solubility was greatly increased and permitted the reliable integration of the sedimentation patterns. [e] Sedimentation studies at this pH are particularly sensitive to total protein concentration and demonstrate some variation from preparation to preparation. [f] Asymmetric peak with trailing edge.

[1] Recent experiments in our laboratory with brushite prepared by H. W. Siegelman of Brookhaven National Laboratory, which has extremely fast flow rates, indicate that exposure to this type of calcium phosphate may not be deleterious to the observed aggregation phenomenon and may be a convenient purification method.

TABLE II: Temperature Studies of Sedimentation.

S. lividus Phycocyanin						*P. calothricoides* Phycocyanin[a]					
pH	Temp (°C)	3 S	7 S	11 S	19 S	pH	Temp (°C)	3 S	7 S	11 S	19 S
6.0[b]	8		64	36		6.0[b]	10		45	50	5
	25		60	40	Trace		25		21	75	4
	49		21	70	10		34		17	80	3
5.0	9	~100	Trace	Trace		5.0	8	17		83	

[a] Data from Scott and Berns (1965). [b] Performed with aliquots from a single sample.

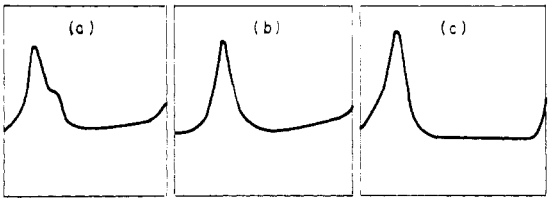

FIGURE 1: Typical sedimentation pattern for *S. lividus* phycocyanin. Sedimentation from left to right: (a) pH 7.0, μ = 0.1 cacodylate, 32 min; (b) pH 8.0, μ = 0.1, phosphate, 46 min; (c) pH 9.0, μ = 0.1, carbonate, 40 min.

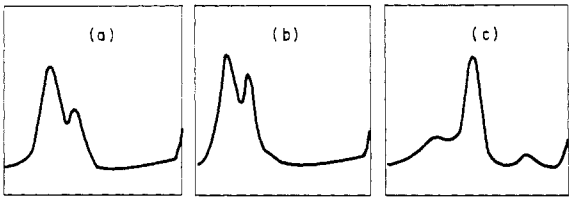

FIGURE 2: Temperature study of sedimentation of *S. lividus* phycocyanin in pH 6.0, μ = 0.1, phosphate. Sedimentation from left to right: (a) 8°, 56 min; (b) 22.5°, 32 min; (c) 49°, 32 min.

Results

General Observations. The ultraviolet and visible absorption spectrum of the purified phycocyanin from *S. lividus* was identical with those observed for purified phycocyanin from *P. calothricoides* and *Ph. luridum*. There was a significantly greater amount of allophycocyanin present in the crude extracts from this alga than in crude extracts of the other Cyanophyta investigated, and the solubility of phycocyanin isolated from this thermophilic alga was observed to be noticeably less than that encountered with *P. calothricoides* and *Ph. luridum*. Solutions (concentration above 4%) of both of these phycocyanins have been prepared in previous work; however, it was not possible to prepare solutions of more than 3 or 4 mg/ml of *S. lividus* phycocyanin in μ = 0.1, pH 5.0 acetate buffer, at room temperature. A solubility of near 15 mg/ml was obtained in sodium phosphate buffer at pH 5.5; however, the solubility in acetate buffers at this pH was far less.

Purification of phycocyanin from *P. calothricoides* and *Ph. luridum* from different harvests has always resulted in homogeneous preparations which, as far as can be ascertained, are extremely reproducible in all observed physical properties. The purification of *S. lividus* phycocyanin is more difficult due to its lower solubility at all pH values. The purification of several different harvests resulted in reproducible behavior, but some variation in sedimentation, particularly at pH 6.0, was observed. Antigenically, all these preparations were identical. It is possible to lower the purification pH to 6.0 and obtain greater amounts of the larger aggregates, but the protein's solubility at pH 6.0 severely limits the utility of this procedure.

pH Sedimentation Dependence. The results of sedimentation studies carried out as a function of pH are listed in Table I. The pH dependence of the sedimentation patterns demonstrates behavior analogous to that observed for phycocyanin from *P. calothricoides* and *Ph. luridum* (Scott and Berns, 1965). As the pH increases, the relative amount of 7S species increases and 11S and 19S species decreases (Table I, Figure 1). The sedimentation pattern in the presence of cacodylic acid buffer at pH 7.0 when compared to that of the pH 7.0 phosphate buffer indicates a relative increase in the amount of 11S species as demonstrated in previous studies with *P. calothricoides*. The temperature dependence of sedimentation is tabulated in Table II. At pH 6.0 11S and 19S species increase as the temperature increases (Figure 2). A few experiments with increased ionic strength appeared to show an increase in the heavier aggregate, 11 S, as reported in our previous work. This effect was not investigated in great detail.

Changes in the area under the several peaks as function of total protein concentration were investigated in the region of concentration of 5 mg/ml to about 15 mg/ml at pH 6.0 at 25°. There was a definite variation in the areas, from 5 to 10%, with the area under the 7S peak increasing with decreasing concentration.

The behavior of *P. calothricoides* phycocyanin in our

previous studies (Scott and Berns, 1965) indicated that at 25° in the concentration region investigated, the areas under the several peaks were apparently independent of total protein concentration. At ~5° the results were analogous to those of the investigation at 25° for *S. lividus* phycocyanin. At pH 6.0, $\mu = 0.1$, we calculated from the sedimentation pattern the relative equilibrium constants for the trimer–hexamer reaction for *P. calothricoides* phycocyanin at 3° and 25° (Scott and Berns, 1965) as 4×10^3 and 4×10^4 l./mole, respectively. A simple calculation of the relative change in concentration of the trimer, or 7S concentration at 25°, *vs.* the same change at 3°, demonstrated that at 25° the 10 mg/ml–20 mg/ml concentration region is a good deal less sensitive for changes in the relative amount of 7S species; much greater increases in 7S concentration are expected in the 1 mg/ml region. At 3°, the change in the equilibrium constant dictates a shift in the concentration region where the variation in concentration of the aggregates is greatest. This shift is a magnitude higher in protein concentration, so that in the 10 mg/ml region, the area changes should become measurable. This would indicate that the sedimentation pattern of *S. lividus* at pH 6.0, $\mu = 0.1$, at 25° should be sensitive to changes in total concentration, since these conditions are apparently comparable to those at pH 6.0, 3°, with *P. calothricoides*.

The sedimentation coefficients presented in Table I and Table II generally represent single determinations; therefore, these values do not correspond exactly to the previously reported extrapolated values for 7 S, 11 S, and 19 S. Most centrifugation examinations were made at concentrations of *ca.* 15–20 mg/ml. Higher concentrations were difficult to obtain. If the sedimentation coefficient concentration plot for *P. calothricoides* phycocyanin is used to investigate whether the sedimentation coefficients for this study correspond to the experimentally determined values for the *P. calothricoides* study, we find the corresponding values to be quite close. Therefore, the designations 3 S, 7 S, 11 S, and 19 S are used. Precipitin lines of partial identity with *P. calothricoides* phycocyanin cited in the immunochemical studies (Figure 3) support the essential identity of the aggregates in *P. calothricoides* and *S. lividus* phycocyanin.

Electrophoresis. Cellulose acetate strip electrophoresis of phycocyanin from *P. calothricoides*, *S. lividus*, and *Ph. luridum* were carried out at pH 4.0, 4.7, and 5.0, $\mu = 0.02$ buffer. At pH 5.0 the *S. lividus* phycocyanin migrated slightly faster than the *P. calothricoides* protein. Both migrated toward the anode. The *Ph. luridum* phycocyanin was essentially immobile. At pH 4.7, the *P. calothricoides* sample migrated slightly toward the anode, the *S. lividus* protein did not move from the origin, and *Ph. luridum* phycocyanin moved significantly toward the cathode. At pH 4.0 all samples migrated toward the cathode with the *Ph. luridum* migrating the farthest. The inference from this qualitative investigation of the electrophoretic behavior of these three proteins is that the isoelectric point of all three proteins is close to a pH of 4.7 with that of *Ph. luridum* probably slightly higher and that of *P. calothricoides* slightly lower.

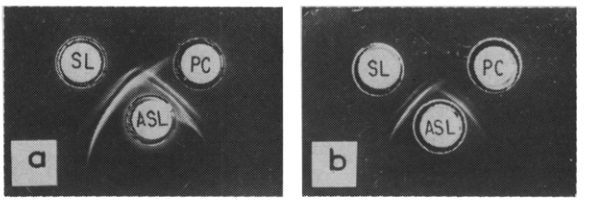

FIGURE 3: Ouchterlony double diffusion studies. ASL, antibody to *S. lividus* phycocyanin; PC, purified *P. calothricoides* phycocyanin; SL, purified *S. lividus* phycocyanin. All lines fluoresce red in the presence of long-wavelength ultraviolet lamp: (a) pH 6.0, $\mu = 0.1$, phosphate; (b) pH 7.0, $\mu = 0.1$, phosphate.

Immunochemical Studies. Ouchterlony double-diffusion studies performed in 0.15 M saline agar at pH 7.0 exhibited the following characteristics. If anti-*P. calothricoides* phycocyanin antisera or anti-*Ph. luridum* phycocyanin antisera were used in the center well and the heterologous and homologous purified or crude antigen preparations were allowed to diffuse *vs.* the antisera, three lines of partial identity were usually evident. Under varying conditions of concentration and pH at times only two lines were found. The intensity of the lines farthest from the antisera well associated with the *S. lividus* phycocyanin was a good deal fainter than that of the corresponding lines with *P. calothricoides* and *Ph. luridum* phycocyanins. These lines would be associated with the slower diffusing or faster sedimenting antigen aggregates. If anti-*S. lividus* phycocyanin sera were used in the center well in the same type of experiment, these slower diffusing antigen lines were much fainter and the fastest diffusing antigen line closest to the antisera well was by far the most intense. If any of the phycocyanin antigens were 1% in sodium dodecyl sulfate the slower diffusing antigen lines were not found to be present. When antisera prepared *vs.* 1% sodium dodecyl sulfate treated *P. calothricoides* phycocyanin were used in diffusion studies, there was a large preponderance of antibody to the fastest diffusing species. Right-angle diffusion plates were used to ascertain the diffusion coefficients of the faster diffusing antigenic species, and diffusion coefficients of the magnitude of 7.5×10^{-7} and 13.5×10^{-7} were found which are in agreement with the values assigned to the 7S and 3S species in our previous studies with *P. calothricoides* phycocyanin (Scott and Berns, 1965).

Ouchterlony diffusion studies were performed with buffers identical with those used in sedimentation studies (pH 6.0, $\mu = 0.1$, phosphate, and pH 7.0, $\mu = 0.1$, phosphate). Both antisera for *P. calothricoides* and *S. lividus* phycocyanin were allowed to diffuse *vs.* the *P. calothricoides* and *S. lividus* phycocyanins as antigens. In Figure 3 one can observe the results at pH 6.0 and 7.0. Two precipitin lines are evident and they demonstrate from the spurring that *S. lividus* and *P. calothricoides* have partial antigenic identity. It is

obvious also that at pH 6.0 the two lines with each antigen have nearly equal intensity, but at pH 7.0 the intensity of the line closest to the antigen well is definitely greater. This may be interpreted as an increase in relative concentration of the faster diffusing antigenic species at pH 7.0, which is consistent with the observed sedimentation behavior in both *P. calothricoides* and *S. lividus* (Table I). In going from pH 6.0 to pH 7.0 we observe a great decrease in 11S species and a corresponding increase in concentration of 7S species; the study using anti-*P. calothricoides* serum yields similar results.

Discussion

The over-all physical behavior of phycocyanin isolated from the thermophilic alga *S. lividus* is identical with that of phycocyanin isolated from Cyanophyta grown at normal temperatures, *P. calothricoides* (Scott and Berns, 1965) (Table III), and *Ph. luridum*. The amount of 7S or trimeric species increases with increasing pH or with deviation from the isoelectric point of 4.7, and is also favored at low ionic strength and at lower temperature. All these characteristics indicate electrostatic interaction. At low pH, high ionic strength, and high temperature, the 11S or hexameric species is favored. The hexamer has been suggested as a species of *in vivo* importance (Scott and Berns, 1965). With *S. lividus* at 49° at pH 6.0 the 11S species becomes predominant and the amount of 19S material increases substantially (Table II). This temperature is close to the 55° culturing temperature of this thermophilic alga. While the trends of the physical behavior of *S. lividus* phycocyanin are analogous to those observed for *P. calothricoides*, the specific distribution of species is quite different. At pH 6.0, $\mu = 0.1$, at 25° the distribution of species is similar to that expected below 10° with *P. calothricoides* (Table II). The distribution at 49° at pH 6.0 is similar to that at 25° for *P. calothricoides*. This is indeed as might be predicted if the hexameric species is an important species *in vivo*. The aggregation phenomena have been displaced 25° exactly as have the growing conditions.

The immunochemical studies support the suggested displacement of equilibria in *S. lividus* to the lower aggregate at room temperature. Immunodiffusion results, the determination of diffusion coefficients, and the presence of precipitin lines of partial identity definitely establish the correlation of the species present in *S. lividus* with those present in *P. calothricoides* and *Ph. luridum*.

The 19S species or dodecamer has also been previously characterized as a species probably important *in vivo*. In these studies at lower temperatures there is either the complete absence of 19S or the presence of only trace amounts (Table II). At higher temperatures the 19S species comprises as much as 10% of the total protein. This would indeed seem to argue for the probable importance of this species *in vivo* and would seem to mitigate against the species being an artifact. In recent additional studies by A. Morgenstern in this laboratory the amount of 19S material present in *P. calothricoides* preparations is greatly increased by purification at pH 6.0. In addition, faster sedimenting material is present; however, exposure to higher pH values and return to pH 5.0 cause loss of most of the 19S material and all faster sedimenting species.

There are several possible explanations for the observed displacement of aggregation equilibria observed in this study. Obviously, a change of isoelectric point could be a possibility, but at present this has not been demonstrated as a fact. The lower solubility of *S. lividus* phycocyanin at pH 5.0 would seem to argue against an isoelectric point lower than 4.7, and might be construed to mean the isoelectric point has increased. Experimental difficulties have not permitted a dependable estimate of the isoelectric point by free solution electrophoresis, although cellulose acetate electrophoresis indicates a point near 4.7. An explanation not inconsistent with the possibility of a change in isoelectric point is an increase in the over-all number of charged or acidic and basic amino acid residues. The amino acid analysis of Berns *et al.* (1964) for *P. calothricoides* and *S. lividus* phycocyanin can be used to demonstrate the most significant differences in amino acid content (Table III). *S. lividus* phycocyanin has an apparent increase in arginine, glutamic acid, and probably tyrosine residues with significantly less serine, glycine, and alanine. The number of charged and polar residues is definitely increased in the thermophilic phycocyanin. Since the glutamic acid residues represent both glutamine and glutamic acid residues, it is not

TABLE III: Amino Acid Content of Phycocyanins.[a]

Amino Acid Residue	*P. calothricoides*	*S. lividus*
Lysine	10	11
Histidine	2	2
Arginine	14	19
Aspartic acid	28	28
Threonine	14	14
Serine	16	10
Glutamic acid	18	30
Proline	8	10
Glycine	20	10
Alanine	40	26
Cystine	1	1
Valine	16	17
Methionine	8	8
Isoleucine	14	16
Leucine	22	23
Tyrosine	10	16
Phenylalanine	8	10

[a] Data from Berns *et al.* (1964); in residues per monomer unit.

profitable at this point to discuss possible differences in isoelectric point from this information. The relative increase in charged and polar residues is at present far more impressive and is consistent with the greater mobility observed at pH 5.0 for the *S. lividus* phycocyanins. This would present a feasible explanation for the increase in the relative amount of trimer or electrostatic aggregate at a lower pH and at room temperature. The increased electrostatic interaction because of the presence of the additional charged and polar residues would result in more mutual repulsion among subunits and greater rupturing of the proposed stable hexamer conformation, and hence the increased amount of what has been designated as trimer.[2] The greater repulsion would require additional thermal energy to overcome and allow the probably more specific forces responsible for the hexamer and higher aggregate formation to become effective, be they hydrophobic, van der Waal's, or specific side-chain interaction.

This postulated mechanism may be clarified to some extent by the suggestion that the hexamer is probably formed from monomer species and the trimer is quite likely an artifact due to the electrostatic repulsion. This is supported by studies of *P. calothricoides* phycocyanin at low temperature (~5°) at pH 5, near the isoelectric point. Under these conditions in the presence of minimal electrostatic repulsion only 3S and 11S peaks are present in a sedimentation experiment.

The suggestion of the importance of electrostatic forces in stabilizing the thermophilic aggregating proteins is of general interest. Lower temperatures increase the effectiveness of the electrostatic interactions, in a fashion somewhat comparable to increasing the pH. It is conceivable that by purifying a protein some 50° below its native environment, irreversible changes may occur as a result of the electrostatic effects. The proper purification temperature would be closer to 50°; however, other obvious difficulties mitigate against using this temperature for long periods. It should, however, be considered that even in proteins from organisms grown at 25°, if purifications are carried out at pH values sufficiently removed from the isoelectric point, lowering the temperature to near 0° may be responsible for perturbations of any aggregation phenomena.

[2] The sedimentation pattern for the 7S peak is usually a great deal more diffuse than that for the 11S peak, leading to the suspicion that it may in fact represent a distribution of different trimers or of dimers, trimers, and tetramers. Electron microscopy (Berns and Edwards, 1965) of the protein is in agreement with this possibility.

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