Comparison of the Biliproteins from Two Strains of the Thermophilic Cyanophyte *Synechococcus lividus*

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C-Phycocyanins from two thermophilic strains of *Synechococcus lividus* that grow within different temperature ranges have been shown to be unlike. The aggregation ability of these two C-phycocyanins in sedimentation-velocity experiments varied dramatically. Surprisingly, the aggregation properties of mesophilic C-phycocyanins were found to lie between those of the two thermophilic proteins. Under identical conditions at pH 7.0, one thermophilic protein (Sy I) was composed of 17S and larger aggregates, whereas the other (Sy III) was an almost homogeneous 6S aggregate. Mesophilic C-phycocyanins have a mixture of 6S, 11S and less stable 17S aggregates under these conditions. Amino acid analysis, absorption spectra, immunochemistry and fluorescence polarization all indicated differences in the composition and properties of the thermophilic proteins, which suggest that they have different modes of adaptation to very high temperatures. Allophycocyanins from the two strains of *S. lividus* were also purified and studied, but unlike the C-phycocyanins no major differences were found between them. Allophycocyanin was homogeneous at pH 6.0, with a sedimentation coefficient of 5.54S and mol.wt. 1.03×10^5 , as determined by sedimentation-equilibrium measurements.

Synechococcus lividus grows at as high a temperature as any known photosynthetic organism (Brock, 1967). It is a blue-green alga and thus contains the protein C-phycocyanin, an accessory photosynthetic pigment. We have extended the observations of Bernis & Scott (1966) on one C-phycocyanin from *S. lividus* and have studied the protein from a second strain, which can grow at considerably higher temperatures than the one studied initially. Unexpectedly, salient differences were found in the C-phycocyanins from these two strains: differences in their aggregation behaviour, visible-absorption spectra, immunochemistry and amino acid composition. In addition, in many experiments their characteristics were unique when compared with those of C-phycocyanins isolated from mesophilic blue-green algae which grow at moderate temperatures.

The properties of several proteins isolated from various thermophilic micro-organisms were reviewed by Singleton & Amelunxen (1973). These thermophilic proteins displayed enzymic properties substantially different from those of the same enzymes isolated from mesophilic organisms. However, no consistent molecular basis for thermophily could be discerned from the existing literature. Continued examination of the same protein from a thermophilic and a closely related mesophilic organism seemed helpful to explain these differences. By using C-phycocyanin, it is possible to emphasize the unusual

protein-protein interactions that occur *in vivo* in the formation of phycobilisomes and that persist to some extent in extracts as smaller aggregates (6, 11, 17 and 20S). These aggregates *in vitro* are most probably intermediates in a complicated process leading to phycobilisome formation.

Some experiments on a second biliprotein, allophycocyanin, from these two strains are also reported. Much less information is available on allophycocyanin (Chapman, 1973; Lazaroff, 1973; Craig & Carr, 1968; Glazer & Cohen-Bazire, 1971; Bennett & Bogorad, 1971; Gantt & Lipschultz, 1973) than on C-phycocyanin (Chapman, 1973; Bernis, 1971; Glazer *et al.*, 1973; Kobayashi *et al.*, 1972).

Experimental

The cultures used were strains of a unicellular cyanophyte identified as *Synechococcus lividus* Copeland. Strain Sy I was an isolate obtained in 1969 by M. R. Edwards from the drainway of a hot pool known as Geyserino in the lower geyser basin of Yellowstone National Park, at a local temperature of 70–72°C. Growth and ultrastructural characteristics of this extremely thermophilic organism (M. R. Edwards, unpublished work) have been found to be similar to strain OH-68-S described by Meeks & Castenholtz (1971). Cells used in the present study were from cultures grown under continuous fluores-

cent light (2000–4000lx) at 59–68°C for 2–4 weeks in a defined synthetic medium [medium D; Table 2, Castenholtz (1970)]. Portions (1 litre) of this medium in Erlenmeyer flasks were each inoculated with 20 ml of a previously prepared culture and stirred manually once or twice a day; a few cultures were stirred continuously with magnetic stirrers. These cultures have not been cloned and are not axenic. An orange filamentous bacterium has been persistently present.

The second strain (Sy III) was a pure culture derived from that previously investigated in our laboratory (Bernis & Scott, 1966; Edwards *et al.*, 1968). Growth conditions were the same as indicated above, except the temperature was 49–53°C. Attempts to grow this strain above 55°C have not been successful.

C-Phycocyanin was extracted from each strain and purified in sodium phosphate buffer, 1.0 M, pH 6.0, to a ratio of E_{620} (Sy III) or E_{610} (Sy I) to E_{280} above 4.5. Fractionations with $(\text{NH}_4)_2\text{SO}_4$, alone or in conjunction with sucrose density gradients and isoelectric focusing, were used in the purification. Discontinuous sucrose gradients were made from 2.0 M-, 1.0 M-, 0.75 M- and 0.5 M-sucrose in phosphate buffer and were centrifuged for 20 h at 140000g and 5°C in a Beckman type 40 rotor.

Isoelectric focusing was particularly important in

purifying allophycocyanin. By using a 110 ml LKB Ampholine column (8101) with a sucrose gradient, a separation of 0.4 pH unit was obtained between C-phycocyanin and allophycocyanin (Fig. 1), which permitted purification of the allophycocyanin. At sufficiently low concentrations, allophycocyanin focused as a closely spaced doublet which was not resolved when fractions were taken. Extinction data for the fractions were determined on a Gilford model 2400 spectrophotometer, and pH was measured on a Beckman SS-1 pH meter. The visible extinction spectrum (Cary 14 spectrophotometer) at pH 6.0 of allophycocyanin showed an E_{652}/E_{620} (Sy III) or E_{652}/E_{610} (Sy I) value of 2.1–2.2 and an E_{652}/E_{280} value of 4.0–4.4.

In general, analytical ultracentrifugation, amino acid analysis and fluorescence measurements were performed as described previously (MacColl *et al.*, 1971a, 1973). A Hamamatsu T.V. photomultiplier tube, R446, was used in the spectrofluorimeter. Extinction spectra were measured on a Cary 14 spectrophotometer at room temperature (24°C). Immunochemical analyses were performed in 1% ion agar at pH 6.0 by the Ouchterlony (1968) double-diffusion procedure with 0.10 ml of either antiserum or antigen in each well.

Fig. 1. Purification of Sy I C-phycocyanin and Sy I allophycocyanin by isoelectric focusing

For details see the text. The isoelectric points are 4.82 and 4.40 respectively. When the allophycocyanin fractions were rerun on the isoelectric-focusing column, no trace of C-phycocyanin was seen. ○, E_{652} ; ●, E_{610} ; △, pH. Fractions are 30 drops each.

EXPLANATION OF PLATE I

Ouchterlony double-diffusion experiment on Sy I C-phycoerythrin and Sy III C-phycoerythrin

Symbols are: A, Sy III C-phycoerythrin at 1.0mg/ml; A2, at 0.50mg/ml; A4, at 0.25mg/ml; A8, at 0.13mg/ml; B, Sy I C-phycoerythrin at 1.0mg/ml; B2, at 0.50mg/ml; B4, at 0.25mg/ml; B8, at 0.13mg/ml; ab 1, antiserum to Sy III C-phycoerythrin. Arrow shows spur. Additional details are in text.

Concentrations were determined from the extinction coefficient at 620nm of C-phycoyanin from *Plectonema calothricoides*, $E_{1\text{cm}}^{1\%} = 60$, in sodium phosphate buffer, I 0.1, pH 6.0.

Results

C-Phycocyanin from the two strains showed differences in both visible extinction spectra and immunochemical properties. At pH 6.0, I 0.1, λ_{max} was 607nm for strain Sy I and 617nm for strain Sy III. A difference spectrum of the two proteins at equal extinction at their respective λ_{max} values revealed the complex differences between them (Fig. 2). Ouchterlony double-diffusion tests (Plate 1) revealed partial identity (spurring) against homologous antiserum.

Although the amino acid compositions of C-phycoyanin from the two strains are similar, differences were noted (Table 1), especially in histidine and arginine. Amino acid data published on strain Sy III C-phycoyanin by Berns & Scott (1966) were recalculated and found to be incorrect. The amino acid compositions were based on mol.wt. 30000, the weight of the 3S monomer for other C-phycoyanins determined by sedimentation equilibrium at pH 3.9 (Kao *et al.*, 1971), and agreed with the sum of the molecular weights of the two chains found by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis in some but not all cases (Glazer & Cohen-Bazire, 1971; Bennett & Bogorad, 1971; O'Carra & Killilea, 1971; Binder *et al.*, 1972; Torjesen & Sletten, 1972). Partial specific volumes were calculated (Cohn & Edsall, 1943) from these data to be 0.733 and 0.734 ml/g for species Sy I and Sy III respectively.

Information on the quaternary structure of these

Fig. 2. Difference spectrum for Sy I C-phycoyanin in reference compartment against Sy III C-phycoyanin in sample compartment

The experiment was performed at room temperature (24°C). The solvent was sodium phosphate buffer, pH 6.0, I 0.1. The spectrum was determined with a Cary 14 spectrophotometer with the extinction of both solutions having a maximum value of 0.93 in the visible region in 1 cm-path-length cells.

proteins was obtained both by sedimentation-velocity studies in the analytical ultracentrifuge and by fluorescence-polarization measurements. Owing to internal energy transfer among the tetrapyrrole chromophores, an inverse relationship exists between the aggregate size of C-phycoyanin and fluorescence polarization (Vernotte, 1971; Goedheer & Birnie, 1965). Fluorescence-polarization measurements over a range of pH values are illustrated (Fig. 3).

Sedimentation results (Figs. 4 and 5) showed that at pH 6.0 phycocyanin from strain Sy I has a significantly distinct state of aggregation, because it has a very large amount of 17S species [compare Fig. 4(a) with Fig. 5(a)]. Phycocyanin from strain Sy I has the same sedimentation pattern in sodium phosphate buffer, at either pH 6.0 or 7.0 (Fig. 4a). In some preparations of Sy I phycocyanin, not only 17S species but also significant quantities of 20S aggregate were observed. Both of these aggregates can be largely eliminated by exposing the protein to a Na_2CO_3 (0.010M)– NaHCO_3 (0.090M)–NaCl

Table 1. Amino acid compositions of C-phycoyanin from Sy I and Sy III strains of *S. lividus*

For details see the text. The values for strain Sy I are averages of 24h, 48h and 72h hydrolyses in 6M-HCl, based on mol.wt. 30000. The values for strain Sy III are averages of 24h, 48h, 72h and 96h hydrolysis in 6M-HCl, based on mol.wt. 30000. N.D., not determined. Values for strain Sy III are re-evaluated data of Berns & Scott (1966).

Residues	Content (mol/mol of C-phycoyanin)			
	Strain Sy I		Strain Sy III	
	Average	Integer	Average	Integer
Asp + Asn	26.7	27	29.6	30
Thr	13.8	14	16.2	16
Glu + Gln	30.0	30	30.0	30
Pro	10.9	11	10.3	10
Gly	20.9	21	21.8	22
Ala	41.5	42	46.3	46
Cys	6.0*	6	2.1	2
Val	16.5	17	19.4	19
Met	6.0	6	7.4	7
Ile	15.1	15	17.1	17
Leu	25.6	26	25.1	25
Tyr	11.8	12	11.9	12
Phe	9.2	9	8.5	9
Lys	9.9	10	11.4	11
His	2.9	3	1.4	1
Arg	19.9	20	15.4	15
Ser†	16.6	17	15.9	16
Trp	N.D.		N.D.	

* Determined as cysteic acid, by method of Spencer & Wold (1969).

† Extrapolated to zero hydrolysis time.

(0.035M) buffer, pH9.0, a harsh treatment for most C-phycocyanins. After this treatment for 3–4 days at 4°C, the solution was dialysed back to pH6.0 or 7.0. The resulting solution was composed predominantly of 6S and 11S aggregates, like the SyIII phycocyanin solution. However, even in this state, where the type of aggregate is qualitatively identical with that found for Sy III phycocyanin, the distribution between the 6S and 11S boundaries is very different for the two proteins [compare Figs. 4(b) and 4(c) with Figs. 5(a) and 5(b)]. The λ_{max} of the absorption spectra (607nm) was the same at pH6.0, both before and after pH9.0 treatment.

It was also observed that even after 4 days at pH9.0, some 17S aggregates of phycocyanin Sy I survived. Experiments on C-phycocyanin from *Phormidium luridum*, purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation so that 70% of its aggregates are above 11S, showed that

pH9.0 treatment converted all aggregates of 11S and larger into 6S and smaller species in less than 1 day at 4°C. Treatment of Sy I phycocyanin for the same duration at pH9.0 resulted in a solution composed of only about one-half 6S species and the rest larger aggregates.

Sy I and Sy III allophycocyanins have at pH6.0 λ_{max} 653 and 652nm respectively, and a single symmetric boundary with $s_{20,w}$ 5.54S measured with the photoelectric scanner also at pH6.0, I 0.1. Since the concentration of the proteins was 0.20mg/ml for this measurement, the sedimentation coefficients were essentially $s_{20,w}^0$. Fluorescence-polarization studies at pH6.0, I 0.1, showed P_{620} to be +0.055 with an s.d. of 0.013 (eight experiments) for Sy I protein and +0.063 with an s.d. of 0.008 (five experiments) for Sy III protein. These results were the same within the limits of experimental error.

Sy I allophycocyanin was studied by sedimentation equilibrium in the analytical ultracentrifuge with scanner optics and the meniscus-depletion method (Yphantis, 1964). Measurements were made on solutions in sodium phosphate buffer, I 0.1, pH6.0, at 21740 and 19160rev./min (AN-F rotor) at 20°C and at 620, 580 and 280nm settings on the monochromator. Data obtained at both speeds and all wavelengths yielded comparable results. These results showed mol.wt. 1.03×10^5 , with s.d. 3%, and a linear plot of r^2 versus $\ln$ concentration. A partial specific volume of 0.738ml/g, calculated from the amino acid composition (R. MacColl, unpublished work), was used in these determinations.

Fig. 3. Fluorescence-polarization (P_{620}) measurements performed on solutions of C-phycocyanin with an extinction of 0.20 at the respective wavelength maxima

For details see the text. ●, Sy I C-phycocyanin; ○, Sy III C-phycocyanin.

Discussion

The C-phycocyanin monomer, as discussed above, is composed of single copies of two different polypeptide chains. At pH6.0, the monomer is observed only at low concentrations (MacColl *et al.*, 1971b),

Fig. 4. Sedimentation-velocity results for Sy I C-phycocyanin

These experiments are performed with schlieren optics at 59780 rev./min in an An-D rotor at 20.0°C. (a) pH6.0, I 0.1; an identical result was obtained at pH7.0; (b) pH6.0, I 0.1, after first treating the protein with sodium bicarbonate buffer, pH9.0, for 4 days; (c) pH7.0, I 0.1, after same treatment as in (b). Each frame was taken 24 min after reaching full speed.

Fig. 5. Sedimentation-velocity results for Sy III C-phycoerythrin

The same conditions as in Fig. 4 were used. (a), pH 6.0, 1.0.1; (b) pH 7.0, 1.0.1. These results are similar to those of Berns & Scott (1966). Strain Sy III solutions were not treated at pH 9.0.

and C-phycoerythrin solutions consist of variable mixtures of higher aggregates (6S, 11S, 17–19S). The 11S species is best characterized and is composed of six monomers (Berns, 1971), and the 6S species has been described as being an aggregate of two or three monomers (Neufeld & Riggs, 1969; Berns, 1971). The protein functions as an accessory pigment in photosynthesis owing to the light-harvesting ability of its tetrapyrrole chromophore, and may also be associated with electron-transport processes (Ilani & Berns, 1972).

The aggregation property of C-phycoerythrin has been examined for a number of mesophilic algae (Berns, 1971; Neufeld & Riggs, 1969; MacColl *et al.*, 1971b; MacColl & Berns, 1973). For example, the equilibrium between the 6S and 11S aggregates under defined conditions of temperature, ionic strength, pH and added small molecules is known and appears to be reasonably constant for all algae tested. However, when the same protein is isolated from an alga that grows under an environmental extreme, the aggregation properties are modified in a manner related to the stress (Berns & Scott, 1966; Kao *et al.*, 1973). Kao *et al.* (1973) demonstrated that C-phycoerythrin from a halophilic alga required high NaCl concentrations to maintain a state of aggregation similar to that of ordinary C-phycoerythrins.

C-Phycoerythrin functions in a cell as an extrinsic membrane protein in the form of large entities called phycobilisomes (Gantt & Conti, 1966). Since assembly of these phycobilisomes is necessary in both mesophiles (Gantt & Conti, 1966; Gray *et al.*, 1973; Kessel *et al.*, 1973) and thermophiles (Edwards *et al.*, 1968; Holt & Edwards, 1972; Edwards & Gantt, 1971), the aggregation properties of C-phycoerythrin may provide a probe of the intrinsic stabilization forces that produce a thermophilic protein. Although

the two C-phycoerythrins were isolated from strains of the same alga and although both strains are thermophilic, the Sy I and Sy III proteins have quite diverse properties. Any unified theory to explain the thermostability must take into account the fact that some properties varied more between the C-phycoerythrins of the two *S. lividus* strains than they did between the thermophilic and mesophilic C-phycoerythrins. The differences between the two thermophilic proteins were small variations in amino acid composition (Table 1), only partial identity in the immunochemical analysis (Plate 1), spectroscopic variations (Fig. 2), and the clear contrast in the relative stability of the aggregates (Figs. 3–5).

The amino acid compositions of Sy I and Sy III C-phycoerythrins have in common a higher content of glutamic acid plus glutamine than do certain of the mesophiles (Table 2). Enolase from two thermophilic bacteria has also been shown by Barnes & Stellwagen (1973) to contain more glutamic acid than enolases from mesophilic sources and, as with Sy I and Sy III C-phycoerythrins, no increase in

Table 2. Comparison of amino acid compositions of some C-phycoerythrins

Each analysis is based on mol.wt. 30000. The *P. calothricoides* analysis is from Berns (1971) and the analysis of *Lyngbya* sp. is from MacColl *et al.* (1971a).

Residues	Content (mol/mol of C-phycoerythrin)			
	<i>Synechococcus lividus</i>		<i>Plectonema calothricoides</i>	<i>Lyngbya</i> sp.
	Strain Sy I	Strain Sy III		
Acidic				
Asp + Asn	27	30	30	33
Glu + Gln	30	30	19	21
Basic				
Lys	10	11	11	11
His	3	1	2	2
Arg	20	15	15	16
Hydroxyl				
Thr	14	16	15	17
Ser	17	16	17	20
Aliphatic				
Gly	21	22	21	23
Ala	41	46	43	42
Val	16	19	17	19
Ile	15	17	15	15
Leu	26	25	24	23
Pro	11	10	9	9
Met	6	7	9	8
Cys	6	2	2	2
Aromatic				
Tyr	12	12	11	13
Phe	9	9	9	8

aspartic acid. Thermostable ferredoxin has been sequenced (Tanaka *et al.*, 1971), and near its active site two residues differ from those in mesophilic ferredoxin: a glutamic acid residue replaces a serine and a glutamine residue replaces an alanine. In many other thermostable enzymes, however, no involvement of glutamic acid has been observed. If glutamic acid is involved in conferring thermostability in these specific cases, its role is most likely its ability to participate in ionic interactions, but aspartic acid might be equally useful if this were the exclusive role. Perhaps the glutamic acid residue confers some important conformational modification on certain thermostable proteins. Generally the amino acid compositions of thermophilic and mesophilic C-phycocyanins are similar (Table 2), as are the results for many related thermophile versus mesophile pairs of proteins (Singleton & Amelunxen, 1973).

The visible extinction spectra of C-phycocyanins at pH 6.0 have λ_{max} 615 nm and higher. The value of 607 nm for Sy I phycocyanin is thus very unusual. Even after treatment at pH 9.0, with dialysis back to pH 6.0, the maximum remains at 607 nm. This constancy shows that the unusual spectrum is not a result of the aggregation state of the protein.

When the aggregation processes of the mesophilic Sy I and Sy III C-phycocyanins are compared, the complexity of the situation is seen. The percentage of 6S C-phycocyanin varies with source at pH 6.0 and 7.0, I 0.1, 20°C, as follows: Sy I < mesophile < Sy III. This analysis applies to solutions where 11S is the only other major component; the Sy I protein is first treated at pH 9.0. In short, both Sy I and Sy III C-phycocyanins are atypical, but they vary in the opposite directions from the mesophile.

Bernis & Scott (1966) showed that whereas at 25°C the Sy III C-phycocyanin has more 6S aggregate than that from a mesophile source, at 50°C the Sy III protein assembles to a state similar to the mesophilic one at 25°C. Their investigations suggested that Sy III protein requires high temperature for complete assembly. This was not found with Sy I protein. In contrast, the thermodynamic stability of 11S aggregate compared with the 6S at pH 6.0, and especially at pH 7.0, is greater than that observed for mesophiles even at 20°C (Fig. 4; MacColl *et al.*, 1971b). In addition, the resistance of the 17S aggregate for disaggregation is greater than that found for the mesophilic protein. At pH 9.0, all aggregates larger than 6S are destroyed for C-phycocyanin from *P. luridum*, whereas under identical conditions (1 day; 4°C) 50% of such aggregates for Sy I C-phycocyanin are retained. Again, in contrast, Sy III C-phycocyanin possessed very little 17S aggregate even at pH 6.0 without being subjected to a pH 9.0 buffer (Fig. 5a).

Fluorescence-polarization experiments (Fig. 3) showed a distinction in aggregate distribution for

Sy I protein versus Sy III even at low concentrations (0.03 mg/ml) and over a range of pH values. Both Sy I and Sy III C-phycocyanins exhibited an increase in polarization at pH 3.9. At this pH the 3S monomer is favoured (Kao *et al.*, 1971; Neufeld & Riggs, 1969; MacColl & Bernis, 1973), but some 11S aggregate is undoubtedly still present in Sy I protein. The wavelength-dependence of the fluorescence polarization for Sy I and Sy III C-phycocyanin in the 560–640 nm region was similar to that observed for other C-phycocyanins (Vernotte, 1971; Teale & Dale, 1970).

The adaptation of C-phycocyanin to high temperature in strain Sy I then is due to the increased relative stability of the larger aggregated forms (11S and greater), in contrast with strain Sy III, where adaptation involves a major temperature-dependence of the aggregation. This augmented stabilization in strain Sy I could allow the forms *in vivo* to continue functioning even at elevated temperatures which denature ordinary proteins. These data suggest that there are at least two alternative conditions for thermophily: (1) high temperature to produce the needed functionality in the protein (a 'thermo-activated' protein); (2) an increase in protein stability, either kinetic or thermodynamic, that discourages thermal denaturation (a 'thermo-resistant' protein).

Both sedimentation-velocity and equilibrium data suggest that, unlike C-phycocyanin, allophycocyanin is a single homogeneous entity at pH 6.0 and low concentration. The homogeneity is indicated by the linear plots of r^2 versus $\ln$ concentration of the equilibrium experiments. The similar molecular weights at both the 620–580 nm and 280 nm wavelengths where many biopolymers absorb also suggest that the preparations are pure. The size of the allophycocyanin was 1.03×10^5 daltons. Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis has shown that allophycocyanin from mesophilic organisms is composed of chains of 1.6×10^4 – 1.7×10^4 daltons (Bennett & Bogorad, 1971; Rice & Briggs, 1973). These results together indicate that the protein is a hexamer. The diffusion coefficient, calculated from the Svedberg equation, was $5.08 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$. The frictional ratio, f/f_{min} , is thus 1.35 (Tanford, 1961). Thus the physical properties of allophycocyanin are very different from those of C-phycocyanin. The functions of allophycocyanin and its relationship to C-phycocyanin are still not substantiated. Lazaroff (1973) has made the interesting proposal that allophycocyanin may be involved in photomorphogenesis.

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