ELSEVIER

Biochimica et Biophysica Acta 1276 (1996) 64–70

BBA Biochimica et Biophysica Acta

Some physical properties of an unusual C-phycocyanin isolated from a photosynthetic thermophile

Mercedes R. Edwards, Robert MacColl *, Leslie E. Eisele

Wadsworth Center, New York State Department of Health, P.O. Box 509, Albany, NY 12201-0509, USA

Received 11 December 1995; accepted 26 March 1996

Abstract

A cyanobacterium, which is a photosynthetic thermophile, has been grown in the laboratory at 66–70°C. This organism, *Synechococcus lividus* (SyI), was isolated from a hot spring in Yellowstone National Park where it grows at 68–73°C. The biliprotein, C-phycocyanin, has been purified, and some of its physical properties studied. When carefully prepared, the protein is obtained in a homogeneous form at about 600 000 molecular weight. Its visible absorption spectrum has a maximum at 608 nm, which is blue shifted to a higher energy maximum than any other known C-phycocyanin. Studies are presented showing that the unique spectrum is not a function of protein aggregation, chemically changed chromophores, number of chromophores, linker polypeptides, temperature of measurement, or of its thermophilic origin. To produce this spectrum, one or more of the chromophores must be affected by apoprotein differently that it is affected in other proteins. The circular dichroism spectra of the protein have been studied, and the aggregation was monitored by fluorescence polarization and gel-filtration column chromatography. A method to prepare SyI monomers was developed. Rods, monomers, trimers, and hexamers were all shown to have blue-shifted absorption maxima. Three other C-phycocyanins — one from a mesophilic and two thermophilic cyanobacteria — were compared with the *S. lividus* (SyI) protein, which was found to be the most thermally stable and most resistent to dissociation.

Keywords: Thermophile; C-Phycocyanin; Biliprotein; Photosynthesis

1. Introduction

C-Phycocyanin is a light-harvesting pigment found in cyanobacteria and certain red algae. It functions by harvesting light in a region of the visible spectrum where chlorophyll *a* has little absorptivity, then this excitation energy migrates from the site of absorption to a reaction center of photosystem II. The absorption of light is achieved by a number of open chain tetrapyrroles (three per monomer), which are covalently attached to apoprotein [1–7].

Data are presented on a C-phycocyanin purified from a strain of the cyanobacterium, *Synechococcus lividus*, isolated from a hot spring at Yellowstone National Park, where it grows at 68–73°C, the highest growth temperature of any organism containing biliproteins. Cultures of this cyanobacterium were grown in our laboratory at 66–70°C. Key points in this study are the unique visible absorption spectrum and much larger, more uniform aggregate state than is typical of C-phycocyanin from other organisms. The methods used include gel-filtration column chromatography, absorption, fluorescence, fluorescence polarization, and circular-dichroism (CD) spectroscopy.

2. Material and methods

Three strains of the unicellular cyanobacterium *S. lividus* [8], plus one strain of the mesophilic filamentous *Phormidium luridum* were used for this study. The *S. lividus* strains have been designated as SyI, SyII, and SyIV according to their growth temperatures, which were approximately at 66–70°, 50–53°, and 54–58°C, respectively.

S. lividus (SyI) was isolated by M.R. Edwards in 1969 from the effluent channel of a thermal alkaline pool called Gyserino located in the lower geyser basin at the Yellowstone National Park, Wyoming. The in situ temperature was 68–73°C, where it was growing on the top of a yellow-green mat (2–4 cm deep) formed by a mesh of

* Corresponding author. Fax: +1 518 4747992; e-mail: robert.maccoll@wadsworth.org

PII S0005-2728(96)00032-1

filamentous bacteria, under a thin layer of running water [9]. The other two strains (SyII and SyIV) were obtained from the culture collection of the Department of Biology, University of Oregon (courtesy of R.W. Castenholz), Y-150-A, our SyII and Y-52-A D(clone 1), our SyIV. *P. luridum* was from the Indiana University culture collection.

All the organisms used were grown in 2-l Erlenmeyer flasks containing 500 ml of Castenholz medium D [10] at pH 7.6–8.4. The flasks were agitated manually (twice daily) or by means of magnetic stirring under a bank of cool-white fluorescent bulbs. A few 100-W incandescent bulbs were used to provide increased red light. Portable floor heaters were used to heat the cultures. High humidity was maintained by a large sink half full with hot water.

The C-phycocyanins of the three strains of *S. lividus* (SyI, SyII and SYIV) were extracted after treatment of the cells with lysozyme or 2–3 passages through a French Press Cell. The cellular mixtures were centrifuged for 15 min at 12 000 rpm. The protein was treated by ammonium sulfate fractionation followed by centrifugation in discontinuous sucrose gradients of 2.0 M (2.0 ml), 1.5 M (1.5 ml), 1.0 M (3 ml), 0.75 M (2 ml), and 0.5 M (1 ml). The gradients were centrifuged for about 20 h at 140 000 × g at 4°C in a Beckman type 40 rotor. C-Phycocyanin was found in the 1 M sucrose near the interface with 1.5 M sucrose and allophycocyanin at the interface of 0.75 M and 1.0 M sucrose. Each preparation was checked for aggregate size by gel-filtration column chromatography as described previously [11]. C-Phycocyanin was considered pure when the absorption ratio of visible maximum to 280 nm was greater than 4.0 [12]. Another criterion of purity was that allophycocyanin was not observed at 650 nm. Samples were selected that were homogenous and had a 600 000 molecular weight. Purified protein that dissociated to hexamers and trimers was used in experiments on the temperature dependence of the CD spectra.

For the experiments with NaSCN, a 4.0 M NaSCN solution was prepared in pH 6.0 buffer and the pH was adjusted back to pH 6.0 using a Hanna pH meter. Solutions of C-phycocyanin were prepared so that there was a constant protein concentration with varying concentrations of NaSCN. Solutions were stored in a refrigerator and measurements were made after one or two days to insure a completed change in the spectra. For absorption studies, a Beckman DU640 spectrophotometer was used. Measurements were made in a 1-cm light path, and the temperature was controlled with a Peltier device. For CD studies, a JASCO J-720 spectropolarimeter was used as described previously [11]. The light paths used for the visible region were 5 or 10 mm, and for the far UV a 0.2 or 0.5 mm light path was used. Temperature was kept at 23°C with circulating water from a Neslab RTE-III refrigerated circulator.

Fluorescence measurements were carried out using a Perkin-Elmer LS50B luminescence spectrophotometer. For emission measurements, the samples were prepared in pH 6.0 buffer having $A = 0.06$ in a 1-cm light path at the visible absorption maxima. The solutions were typically excited at 590 nm using 8-nm slits for both excitation and emission. The polarization (p) was obtained as described previously [4].

The effect of urea on the proteins was determined by dialysis of a small volume of protein into a large volume of 10 M urea, pH 2.5, overnight at ambient temperature. After recording the spectra in 10 M urea, pH 2.5, the solutions were dialyzed from the urea into pH 6.0, sodium phosphate, 0.1 ionic strength buffer.

3. Results and discussion

3.1. Unusual characteristics of SyI C-phycocyanin

Purification of C-phycocyanin from SyI, the highest-temperature strain of *S. lividus*, produced a protein with an absorbance spectrum resembling that of other C-phycocyanins (Fig. 1), but differing in the wavelength maximum (Fig. 1B). A maximum at 608–609 nm is blue-shifted relative to other C-phycocyanins, whose maxima may vary from 615 to 622 nm depending on pH, protein concentration, and other factors [1].

A second unusual characteristic is the aggregation state of the purified protein. Gel-filtration column-chromatography experiments (Fig. 2) showed that the protein, when purified carefully, has a molecular weight of about 600 000 and this preparation is almost homogenous. Ordinarily, purified C-phycocyanins in the presence of linker polypeptides are obtained as complex solutions containing

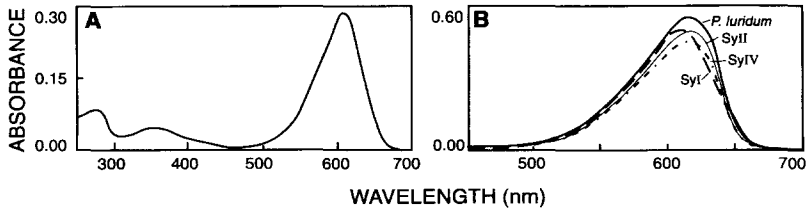

Fig. 1. Absorption spectrum of C-phycocyanin from *S. lividus* (SyI). Panel A is the spectrum of C-phycocyanin from *S. lividus* (SyI); panel B is the comparison of the visible spectra of C-phycocyanins from *S. lividus* (SyI), *P. luridum* and two other *S. lividus* strains SyII and SyIV. Buffer was pH 6.0, 0.1 ionic strength, sodium phosphate, temperature was 23°C.

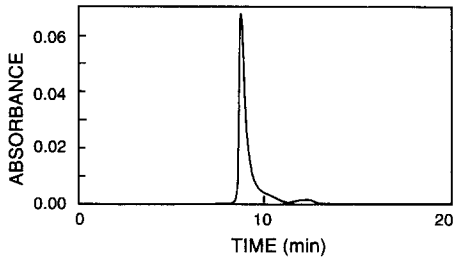

Fig. 2. Gel-filtration column chromatography experiment of C-phycocyanin from *S. lividus* (SyI). Buffer was pH 6.0, sodium phosphate. Elution was monitored at 610 nm.

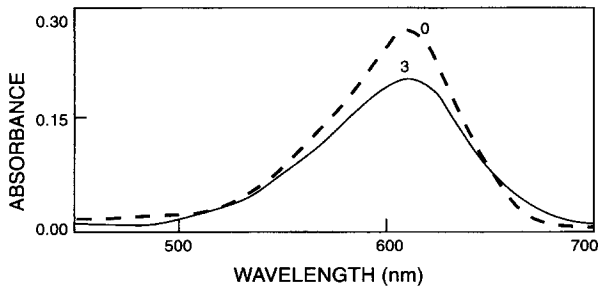

Fig. 4. Absorption spectra for C-phycocyanin from *S. lividus* (SyI) in 0 and 3.0 M NaSCN, pH 6.0. Numbers on spectra refer to molar concentrations of NaSCN.

monomers, trimers, hexamers, and some amounts of larger aggregates [4]. The 600 000 molecular weight of SyI when divided by the monomer molecular weight of 35 000 suggests that it is a grouping of 3 hexamers ($3 \times 6 \times 35\,000 = 630\,000$). The phycobilisome rods vary in length with 2–4 hexamers being common. It is concluded that the 600 000 molecular weight aggregates are probably the intact rods from the phycobilisomes, which have dissociated from the cores.

3.2. Effect of NaSCN on SyI C-phycocyanin

It may be that the absorption maximum at 608 nm results from the unusually large aggregation state of the protein. To investigate this possibility, the dissociation of C-phycocyanin $(\alpha_6\beta_6)_3$ was carried out. NaSCN, a chaotropic salt, has been used extensively in the study of C-phycocyanin because of its capability at 1–2 M to dissociate the aggregates totally to monomers for protein from all organisms so far tested [12]. Monomers have a characteristic polarization at the red edge of their spectrum of +0.35–0.4 [4]. Aggregates will have correspondingly lower polarizations because of energy migration among the chromophores. C-Phycocyanins from three sources, one mesophilic and two thermophilic, illustrate monomer formation at 1 M NaSCN (Fig. 3). The fluorescence polarization of C-phycocyanin of *S. lividus* (SyI) as a function of NaSCN is different (Fig. 3) from the results found for other C-phycocyanins. The very low polarization in the absence of NaSCN is expected because of the large size of the aggregates, but 1 M NaSCN does not produce a polarization indicative of monomers. At 2–3 M NaSCN, the polarization does approach +0.4, and the C-phycocyanin may be considered monomeric. Since the absorption maximum in 3.0 M NaSCN is red-shifted only very slightly to about 609–610 nm and not 615 nm, the unusual maximum does not appear to be a characteristic of rods (Fig. 4). Monomers lack linker polypeptides [4], and, therefore, linkers are eliminated as causes for the unique spectrum.

NaSCN could possibly affect the spectra of the tetrapyrroles, so the NaSCN was dialyzed away and the C-phycocyanin analyzed by gel-filtration column chromatography (Fig. 5A). The solutions previously in 1, 2, and 3 M NaSCN now eluted with all of them showing two bands: the slower is monomers and the faster probably hexamers with possibly some trimers mixed in. The lower aggregation of the protein after thiocyanate treatment probably reflects the loss of certain linker polypeptides [4]. For normal C-phycocyanins, the absorption maxima would be about 620 nm for mixtures of these aggregates, but for SyI

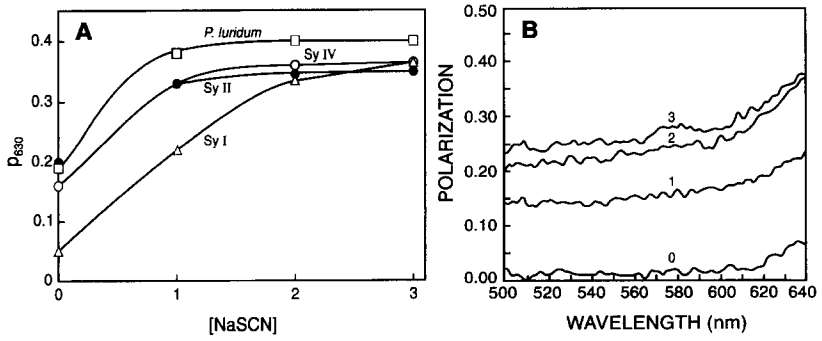

Fig. 3. Fluorescence polarization of C-phycocyanins from *S. lividus* (SyI, II, and IV) and *P. luridum* as a function of NaSCN concentration. Buffer was pH 6.0, sodium phosphate. Concentrations were identical for all, $A = 0.06$ at the maxima in a 1-cm light path. Panel A is p630 versus [NaSCN]; panel B is p versus wavelength for C-phycocyanin from *S. lividus* (SyI). In panel B, the numbers refer to the molar concentrations of NaSCN.

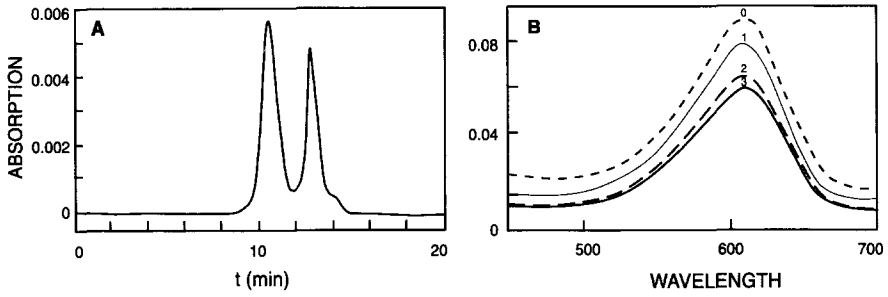

Fig. 5. Absorption spectra and gel-filtration column chromatography for C-phycocyanin from *S. lividus* (SyI) previously treated with NaSCN. Protein solutions were first treated with NaSCN and the salt was then dialyzed away. The chromatogram, panel A, is shown for a previously 3.0 M NaSCN sample. Samples are in pH 6.0 buffer. Numbers on spectra in panel B refer to previous NaSCN concentration.

C-phycocyanin the maxima were at 609 nm (Fig. 5B). These results again show that the unusual blue-shifted maximum of this protein is not caused by assembly into rods.

3.3. CD spectrum of SyI C-phycocyanin

The visible CD spectra of three C-phycocyanins (SyI, SyII and SyIV) were obtained. The results suggest that the maxima of the three different chromophores are fairly similar when one C-phycocyanin is compared with another (Fig. 6), and the shift in the absorption maxima for the SyI

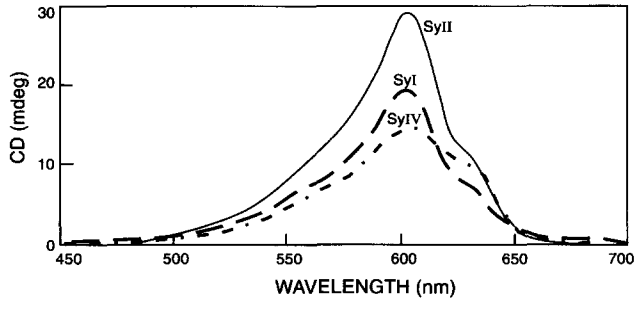

Fig. 6. Visible CD spectra of C-phycocyanin *S. lividus* (SyI, II, and IV). Buffer was pH 6.0 sodium phosphate. Temperature was 23°C. SyI solution had $A_{608} = 1.1$; SyII solution had $A_{608} = 0.85$; SyIV solution had $A_{608} = 0.99$, all in 1-cm light paths. Light paths used for CD were 10 mm (Sy II) and 5 mm (Sy I and Sy IV).

protein may be caused by changes in the oscillator strengths of the chromophores.

3.4. Possible causes of the unique SyI C-phycocyanin spectrum

The spectra of monomeric C-phycocyanin is different for SyI (Table 1). There are four possibilities: the chromophores are chemically different for the SyI protein; there are a different number of chromophores; the temperature of the measurement is responsible; or the apoprotein of SyI interacts differently with the chromophores than the apoproteins of the other C-phycocyanins. To differentiate between these possibilities, the SyI and the *P. luridum* C-phycocyanins were both subjected to extreme denaturing conditions, 10 M urea, pH 2.5. The objective was to minimize as much as possible the effect of apoprotein on the chromophores. Under these conditions, the spectra of the two proteins are identical (Fig. 7), and the chromophores of both proteins are phycocyanobilins [4]. The increase in the intensity of the near-UV band compared with the visible band is the expected result for a change in the chromophores from linear to cyclic conformation [4]. The shift to 662 nm is caused by protonated chromophores [4]. Since both proteins are at the same concentration and both visible spectra have the same oscillator strength, it can be concluded that both C-phycocyanins have three

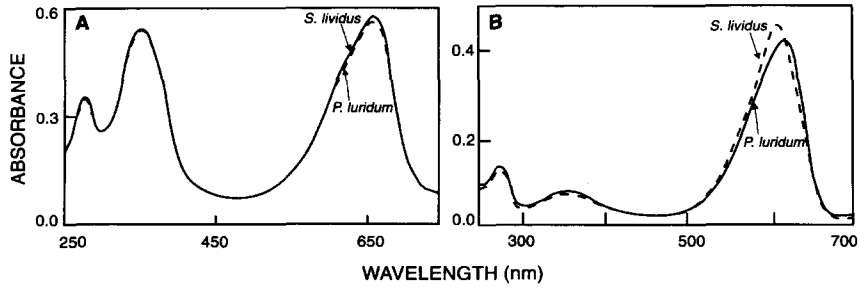

Fig. 7. Absorption spectra of C-phycocyanins from *S. lividus* (SyI) and *P. luridum* in 10 M urea, pH 2.5. Temperature was 23°C. Panel A proteins in 10 M urea, pH 2.5; panel B, protein solutions dialyzed back to pH 6.0, 0.1 ionic strength, sodium phosphate buffer. Protein concentrations were the same.

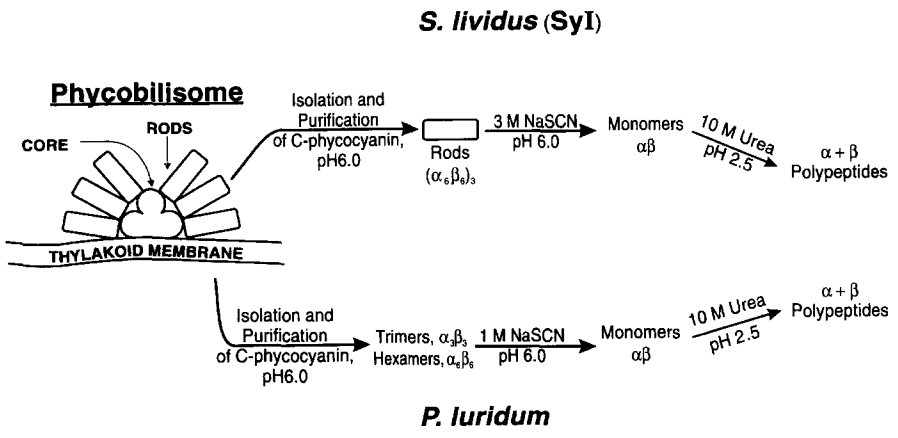

Fig. 8. Scheme for dissociations of phycobilisomes and C-phycocyanins from *S. lividus* (SyI) and *P. luridum*. Edwards and Gantt [17] have shown that SyI phycobilisomes have a similar morphology as other cyanobacteria [4].

chromophores per monomer (Fig. 8). When these solutions were dialyzed back to pH 6.0 buffer without urea, the absorption maxima returned to 608 and 617 nm for *S. lividus* (SyI) and *P. luridum* C-phycocyanin, respectively.

The absorption spectrum of SyI C-phycocyanin with maximum at 608 nm was obtained at 23°C (Fig. 1). When it is examined at 70°C, the absorption maximum is 609 nm (unpublished result).

Solutions of SyI C-phycocyanin containing monomers, trimers and hexamers were run on a gel-filtration column. The photodiode array detector was used to obtain the approximate maxima of these aggregates (Fig. 9, Table 1). Each aggregate of C-phycocyanin from *S. lividus* (SyI) is blue-shifted when compared with other C-phycocyanins.

Table 1
The absorption maxima of C-phycocyanin aggregates and subunits *

Aggregate	Maxima(nm)		
	S. lividus (SyI)	*P. luridum* [15]	*A. nidulans* [16]
Phycobilisomes		627 *	
Rods, $(\alpha_6\beta_6)_3$	608		
Hexamers, $\alpha_6\beta_6$	609 * *	622	621
Trimers, $\alpha_3\beta_3$	609 * *	618	
Monomer, αβ *	607 * * –610 * * *	615	615
α			620
β			608

* A phycobilisome contains rods and a core. Presence of core proteins will red-shift the maximum of C-phycocyanin. Debreczeny et al. [13] found the individual chromophores on monomeric C-phycocyanin to have absorption maxima at 600–602, 624, and 626–628 nm. The α-subunit had the 624-nm maximum. Demidov and Mimuro [14] found monomer maxima at 596, 618, and 625 nm. The 618-nm maximum was for the α-subunit.

* * The values were obtained from gel-filtration column-chromatography experiments using a photodiode array detector. This method will allow the study of each aggregate as it passes off the column, but will be less accurate than using a spectrophotometer. In particular, the monomer concentration of these solution was very small, and there may be error in the 607-nm result.

* * * The monomers of *S. lividus* (SyI) in 3 M NaSCN, pH 6.0 have an absorption maximum at 609–610 nm as obtained with a free-standing spectrophotometer.

3.5. Helix, thermal stability, energy transfer

The CD spectrum of SyI C-phycocyanin was obtained in the far UV region (Fig. 10). The spectrum suggested that the protein has a high α-helix content. This is consistent with the finding for all other biliproteins so far studied [11].

SyI C-Phycocyanin was studied as a function of temperature using CD (Fig. 10). A comparison of CD at 222 nm with protein from *P. luridum* demonstrated a salient increase in thermostability for the SyI protein (Fig. 11). Thermophilic C-phycocyanins, SyII and SyIV, are also much more stable than mesophilic protein, but less stable than SyI protein.

It is not clear that this spectral change to 608 nm would impart any ecological advantages to the organism. C-Phycocyanins transfer excitons to allophycocyanin. The spectral overlap between the emission of C-phycocyanin and the absorption of allophycocyanin is an important factor in the efficiency of this energy transfer. The absorp-

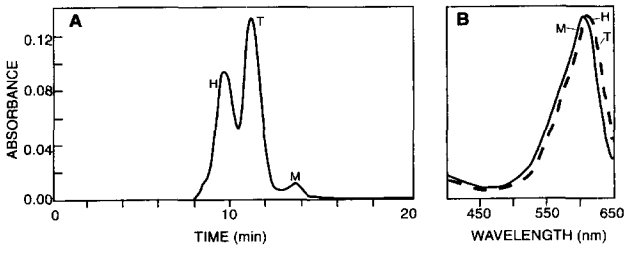

Fig. 9. Absorption spectra and gel-filtration column chromatography of C-phycocyanin aggregates from *S. lividus* (SyI). Panel A is the chromatogram showing hexamers (H), trimers (T), and monomers (M). Panel B is the absorption spectra obtained by using the photodiode array detector.

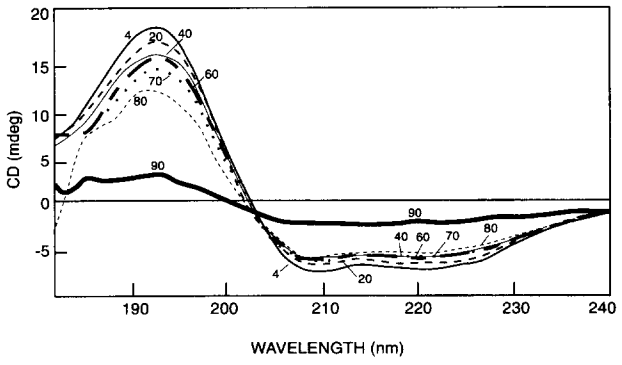

Fig. 10. CD spectra of C-phycocyanin from *S. lividus* (SyI) in the far UV. Numbers on spectra refer to temperature (°C) of the samples. Light path was 0.5 mm. The solution had an $A_{608} = 0.50$. Samples were maintained at each temperature for 15 min before spectra were recorded.

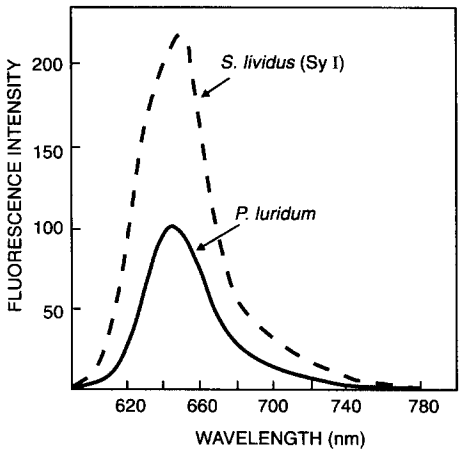

Fig. 12. Fluorescence emission spectra of C-phycocyanin from *S. lividus* (SyI) and *P. luridum*. Solutions were pH 6.0 and excitation was at 590 nm.

tion spectrum of allophycocyanin from *S. lividus* (SyI) is identical to that found in other allophycocyanins [18,19]. A blue-shifted absorption for C-phycocyanin would, if it resulted in a blue-shifted emission, lower the spectral overlap with allophycocyanin. However, the emission of SyI C-phycocyanin is slightly red shifted compared to other C-phycocyanins causing better overlap (Fig. 12). For C-phycocyanins, the emission maxima are 646 and 649 nm, for *P. luridum* and *S. lividus* (SyI), respectively. This means, even though the C-phycocyanin (SyI) absorption spectrum is blue shifted, the lowest-energy chromophore responsible for transfer to allophycocyanin probably is not shifted. This conclusion agrees with the visible CD spectra (Fig. 6).

Factors eliminated for causing the blue shift in the absorption spectrum of SyI C-phycocyanin are the number and chemical composition of the chromophores, protein aggregation, linker polypeptides, measurement temperature, and its isolation from a thermophile (Fig. 1B). A most likely cause would be the interaction of different amino acids with one or more chromophores.

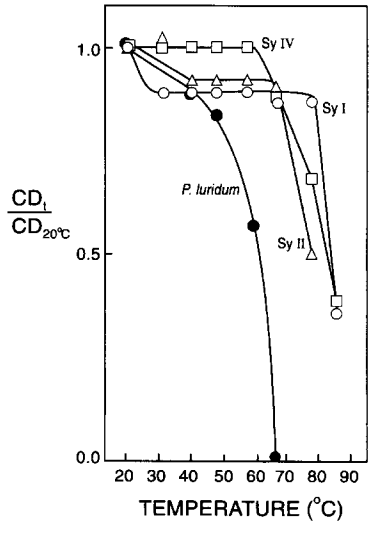

Fig. 11. CD at 222 nm versus temperature for C-phycocyanins from *S. lividus* (SyI (○), II (△), and IV(□) and *P. luridum* (●).

Acknowledgements

Our studies at Yellowstone National Park would not have been possible without the guidance and interest of Dr. Thomas Brock and the use of his laboratory facilities at West Yellowstone, Montana. We are indebted to him. Our thanks are also due to Louise Brock, Dr. William Ghiorse, Dr. Martha Mulks, Inge Panko, Carol Haaksma, Robert J. Bendon, and Edwin C. Williams, whose assistance in this study was indispensable. We also thank Dr. Rami Almog for his most valuable suggestions.

References

[1] Berns, D.S. and MacColl, R. (1989) Chem. Rev. 89, 807–825.
[2] Gantt E. (1979) in Biochemistry and Physiology of Protozoa (Levandowsky, M. and Hutner, S.A., eds.), 2nd Edn., Vol. 1, p. 121, Academic Press, New York.
[3] Holzwarth, A.R. (1991) Physiol. Plant. 83, 518–528.
[4] MacColl, R. and Guard-Friar, D. (1987) Phycobiliproteins, CRC Press, Boca Raton, FL.
[5] Moerschel, E. (1991) Photosynthesis 25, 137–144.
[6] Scheer, H. (1981) Angew. Chem. Int. Ed. 20, 241–261.
[7] Zuber, H. (1987) in the Light Reactions (Barber, J., ed.), pp. 197–259, Elsevier Science, Amsterdam.
[8] Copeland, J.J. (1936) Ann. NY Acad. Sci. 229–341.
[9] Brock, T.D. (1978) Thermophilic Microorganisms and Life at High Temperature, Springer-Verlag, New York.
[10] Castenholz, R.W. (1975) Limnology Oceanography 20, 305–522.
[11] MacColl, R., Malak, H., Cipollo, J., Label, B., Ricci, G., MacColl, D. and Eisele, L.E. (1995) J. Biol. Chem. 270, 27555–27561.
[12] MacColl, R., Berns, D.S. and Koven, N.L. (1971) Arch. Biochem. Biophys. 146, 477–482.

[13] Debreczeny, M.P., Sauer, K., Zhou J. and Bryant, D.A., (1993) J. Phys. Chem. 97, 9852–9862.
[14] Demidov, A.A. and Mimuro, M. (1995) Biophys. J. 68, 1500–1506.
[15] MacColl, R. and Berns, D.S. (1981) Isr. J. Chem. 21, 296–300.
[16] Glazer, A.N., Fang, S. and Brown, D.M. (1973) J. Biol. Chem. 248, 5676–5685.
[17] Edwards, M.R. and Gantt, E. (1971) J. Cell Biol. 50, 896–900.
[18] MacColl, R., Edwards, M.R. and Haaksma, C. (1978) Biophys. Chem. 8, 369–376.
[19] MacColl, R., Edwards, M.R., Mulks, M.H. and Berns, D.S. (1974) Biochem. J. 141, 419–425.