ELSEVIER

Biochimica et Biophysica Acta 1321 (1997) 157–164

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Thermophilic C-phycocyanin: effect of temperature, monomer stability, and structure

Mercedes R. Edwards [a], Charles Hauer [a,b], Robert F. Stack [a], Leslie E. Eisele [a], Robert MacColl [a,b,*]

[a] *Wadsworth Center, New York State Department of Health, P.O. Box 509, Albany, NY 12201-0509, USA*
[b] *University at Albany (SUNY), Department of Biomedical Sciences, P.O. Box 509, Albany, NY 12201-0509, USA*

Received 10 March 1997; revised 30 May 1997; accepted 30 May 1997

Abstract

C-Phycocyanin has been purified from *Synechococcus lividus* (SyI), which grows between 66 and 73°C at the highest temperature of any cyanobacterium and of any biliprotein-containing organism. The protein was examined at its physiological temperature, 70°C, in order to compare with its properties at 20°C. The protein was found to have virtually identical properties of light harvesting, bilin conformation, fluorescence, and secondary protein structure at both temperatures. The unique absorption maximum of SyI protein was maintained at 70°C. Using the bilins as built-in reporter groups, the response is to resist, over a wide temperature range (10–80°C), the denaturation that would occur in mesophiles, rather than exhibit special adaptation to 70°C. C-Phycocyanin from another thermophile, *S. lividus* (SyIII), behaved differently. The SyI protein was 'temperature-resistant', and the other 'cold-dissociated'. In addition, an early assembly step toward the phycobilisome is the formation of monomers ($\alpha\beta$) from bilin-bearing, α and β polypeptides. Unlike larger aggregates, C-phycocyanin monomers from SyI are found to denature between 60°C and 70°C. The instability of monomers at their physiological temperature suggests that they either rapidly aggregate to avoid denaturation, or they are protected from denaturation by some agent. Monomers of the thermophile were, however, much more stable against high temperature than the monomers from mesophiles. The structure of SyI C-phycocyanin and its phycobilisomes were shown to be similar to those of mesophiles. © 1997 Elsevier Science B.V.

Keywords: Thermophile; C-Phycocyanin; Biliprotein monomer; Photosynthesis

1. Introduction

Biliproteins are light-harvesting pigments normally associated with photosystem II. In cyanobacteria and red algae, they assemble into large structures of biliproteins and linker polypeptides, the phycobilisomes, which have an allophycocyanin core with rods of phycocyanin and sometimes phycoerythrin or phycoerythrocyanin. Linker polypeptides produce the higher states in the assembly of the biliproteins. These biliproteins have characteristic numbers and types of open-chained bilins covalently attached to the apoproteins [1–7].

Synechococcus lividus, strain SyI, grows at the

* Corresponding author. Fax: +1 (518) 474-7992; E-mail: robert.maccoll@wadsworth.org

PII S0005-2728(97)00056-X

highest temperature of any known cyanobacterium. It contains C-phycocyanin and allophycocyanin. Phycoerythrin has never been observed in a thermophilic organism. The C-phycocyanin and allophycocyanin of SyI have a salient ability to resist thermal denaturation [8–10]. The purified SyI C-phycocyanin denatures at 90°C. It has a uniquely blue-shifted absorption maximum at 608 nm compared with normal C-phycocyanin maxima between 615 and 622 nm [10].

The studies on thermophilic C-phycocyanin and allophycocyanin have typically been carried out near normal ambient temperatures, around 20–25°C. Thermophiles are heat-tolerant organisms existing below about 90°C, and hyperthermophiles exist above 90°C. Higher temperatures have been used only to test the denaturation of these proteins. It is of interest to examine some of this SyI protein's properties at 70°C to determine whether there is some particular adjustment to the physiological condition. In addition, some proteins exhibit cold denaturation — they denature on cooling ([11] and references within), and this property should be probed for C-phycocyanin (SyI). This C-phycocyanin has an unusual absorption spectrum, and it is of interest to determine if it is maintained at 70°C. These experiments include the absorption, fluorescence, and circular dichroism (CD) spectra of the bilins, and the determination of the far UV CD spectrum of this protein to determine its polypeptide secondary structure at 20°C and 70°C. The SyI phycobilisomes, dissociation products, and individual polypeptides were examined.

The assembly of phycobilisomes must proceed through many steps beginning with the synthesis of the α and β polypeptides of each biliprotein. The initial event in the assembly of these two polypeptides is the formation of monomers (αβ). In addition to C-phycocyanin (SyI) monomers, monomers from a mesophilic C-phycocyanin from a cyanobacterium and mesophilic phycocyanin 645 from a cryptomonad were examined. Their thermal stabilities as monomers and aggregates were measured for the first time to compare relative monomer stability for a selection of biliproteins.

C-Phycocyanin from a second thermophilic cyanobacterium, *S. lividus* (SyIII), was also studied to demonstrate a different behavior from that shown by the SyI protein.

2. Materials and methods

S. lividus (SyI) was isolated in 1969 from a thermal pool as described previously [10]. It was cultured in the laboratory at 66–70°C, and its C-phycocyanin was isolated and purified [10]. The criterion of purity was $A_{608}/A_{280} > 4.0$ [12]. Purified protein was stored under 50% saturated ammonium sulfate in the cold. When needed, protein was dialyzed into pH 6.0, 0.1 ionic strength, sodium phosphate buffer.

Phormidium luridum was used as a source of mesophilic C-phycocyanin [10], and phycocyanin 645 was obtained from *Chroomonas* species [13]. Methods have been developed to dissociate all these proteins to monomers (αβ), while not producing separated α and β polypeptides [10,12,13]. The methods are: for C-phycocyanin (SyI) at pH 6.0, 2.0–3.0 M NaSCN; for C-phycocyanin of *P. luridum* at pH 6.0, 1.0 M NaSCN; for phycocyanin 645, pH 4.0, 0.1 M, sodium acetate.

S. lividus, strain SyIII, was grown at 55°C, and the C-phycocyanin isolated.

For stability studies with NaSCN, controls using NaCl at the same molarities were employed because NaCl does not dissociate biliproteins [12]. In some cases, 2 M NaCl has been shown to increase C-phycocyanin aggregation [4]. The absorption and CD spectra were obtained as described previously [13].

The purified C-phycocyanin of SyI was studied by electron microscopy using negative stain. Tiny drops of a diluted solution of this biliprotein were spread on grids coated with a thin carbon film and stained with saturated uranyl acetate. Also, thin sections of SyI cells were prepared by standard procedures. Grids were examined in a Philips 301 microscope at 80 kV.

Gel-filtration column chromatography was used to establish the aggregation of SyI C-phycocyanin after treatment with the dissociating salt, 1 M NaSCN. The C-phycocyanin was treated overnight with 1 M NaSCN, and the salt was then dialyzed away. A mixture of molecular mass standards were used to calibrate the column: gamma globulin, 158 000; ovalbumin, 44 000; myoglobin, 17 000; and vitamin B-12, 1350. The buffer used was pH 6.0, 0.1 ionic strength, sodium phosphate.

The α and β protein subunits were separated by reverse phase HPLC on a 1 × 150 mm VYDAC C4 column (Hespernia, CA), with an acetonitrile/0.5%

TFA eluent buffer. Electrospray ionization spectra were obtained with a Finnigan TSQ (San Jose, CA) mass spectrometer. Samples were infused at 0.5 μl/min, spectra were acquired over an m/z 500–2000 range per 3-s scan, and the results deconvoluted into mass values.

3. Results and discussion

3.1. 70°C behavior

The near UV-visible absorption spectrum of aggregated C-phycocyanin (SyI) at 70°C resembled that at 20°C (Fig. 1A). There was a 1-nm red shift at 70°C and a slight loss in absorption. Both these changes were totally reversed when the temperature was returned to 20°C (Fig. 1A). These changes are not significant in terms of C-phycocyanin function. The visible absorption maximum at 70°C shifted very minimally to about 609 nm, but still this was blue-shifted compared with all the other C-phycocyanins. The A_{370} did not change at 70°C. The results at A_{370} indicated that there were no changes in bilin conformation at 70°C [4], because a change from linear to cyclic bilin conformation produces an increase in 370-nm absorption.

In the UV between 180 and 260 nm, the CD spectrum can be used to estimate protein secondary structure [14]. At 70°C and 20°C, the UV CD from 180 to 260 nm were similar (Fig. 1B). Calculations indicated that the secondary structures at 20°C were 54.7% α helix, 80.5% β sheet, 19.0% turn, and 16.9% other; and at 70°C, there was 48.8% α helix, 11.7% β sheet, 19.6% turn, and 19.8% other.

For C-phycocyanin, excitation energy is transferred from bilin to bilin apparently by Förster dipole–dipole resonance coupling [15]. The overlap of the fluorescence emission of a donor with the absorption of an acceptor is important in this mechanism. The fluorescence emission of C-phycocyanin (SyI) at 70°C is 650 nm (data not shown), and this maximum is the same as at 20°C.

Visible CD at 20°C and 70°C showed strong similarity (Fig. 1C). The bilins are in similar states at both temperatures. Visible CD is very sensitive to the interaction among bilins. In other biliprotein studies, the bilins have been reported on their tertiary structure changes [16].

There are no special spectroscopic properties of C-phycocyanin (SyI) at 70°C. The only unique event is that the protein is quite stable at this temperature where other C-phycocyanins would denature [10]. Its light-harvesting and energy transfer faculties are similar at 70°C and 20°C, as are the conformations of its apoprotein and bilins. For SyI C-phycocyanin, there are only very small changes between 20°C and 70°C (Fig. 1). The protein, therefore, resisted the denaturing effect of high temperature rather than yielding a more functional form of the protein at these high physiological temperatures. The protein did not cold denature (Fig. 1). At 10°C, the protein's visible spectrum was the same as at 20°C.

C-Phycocyanin from SyI has one property, which is not related to thermophilicity, that is unique. It has a visible absorption maximum at 607–609 nm, compared to other C-phycocyanins both mesophiles and thermophiles whose absorption maxima range between 615 and 622 nm [10]. All these absorption spectra were taken at ambient temperatures, around

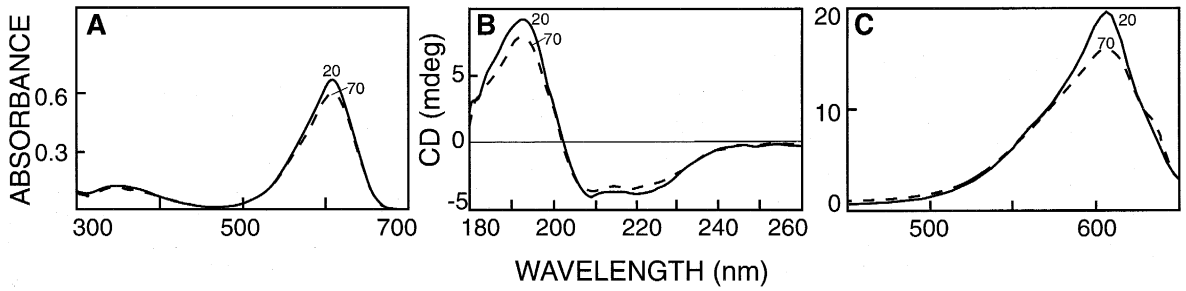

Fig. 1. Studies on C-phycocyanin (SyI) as a function of temperature. A: absorption spectra at 20°C and 70°C; B: CD in the UV at 20°C and 70°C; C: CD in the visible at 20°C and 70°C. In panel A, the reversal of a 70°C sample to 20°C is shown, and when a sample at 70°C was returned to 20°C the spectrum exactly overlaid the original 20°C spectrum.

20–25°C. The question then was whether the 608-nm maximum would red shift at 70°C. It did not shift significantly and was observed at both 20°C and 70°C (Fig. 1A). The maximum at 608 nm was, therefore, the functional state of the protein. Several possible causes for the 608-nm maximum have been eliminated, and the possibility of a unique bilin and amino acid interaction has been forwarded [10]. The Stokes shift for this C-phycocyanin is probably the same as for other C-phycocyanins since emission occurs from a red-shifted bilin that is similar in all cases.

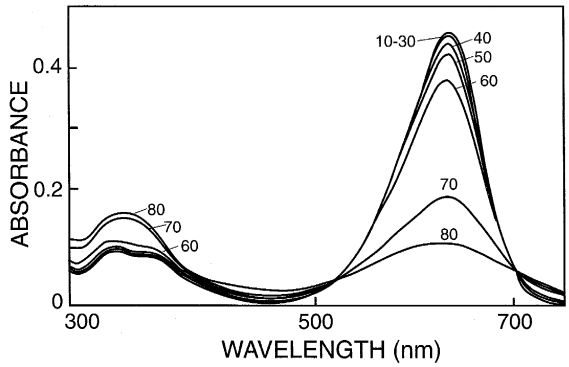

Fig. 3. Effects of temperature on the visible and near-UV absorption spectra of monomers of C-phycocyanin (SyI). Numbers on curves refer to temperatures in °C.

3.2. Monomers

Monomer (αβ) units of biliproteins must exist at some point in the assembly of all biliproteins. Monomers of phycocyanin 645 have a similar secondary structure to dimers [13], and, therefore, secondary structure could be established early in the maturation of this biliprotein. The thermal stability of biliprotein monomers is unknown, since earlier studies have naturally focused on the important aggregated forms [4,10,17–22].

Monomers of SyI C-phycocyanin denaturated at lower temperatures than that of the aggregates (Fig. 2C). At 70°C, monomers showed complete disorder. The aggregated C-phycocyanin was virtually unchanged at 70°C and only slightly hypochromic at 80°C (Fig. 2C).

To determine whether monomers of other biliproteins were likewise less stable than aggregates, C-phycocyanin from *P. luridum* and phycocyanin 645 from *Chroomonas* were examined as well (Fig. 2B and Fig. 2A, respectively). A loss of stability was observed for all three monomers (Fig. 2).

The thermal disordering of SyI monomers suggested that these monomers are not stable at their physiological temperatures (66–73°C). In addition to the loss of visible absorption at 70°C, the near-UV band intensifies at 70°C (Fig. 3). This increase in the

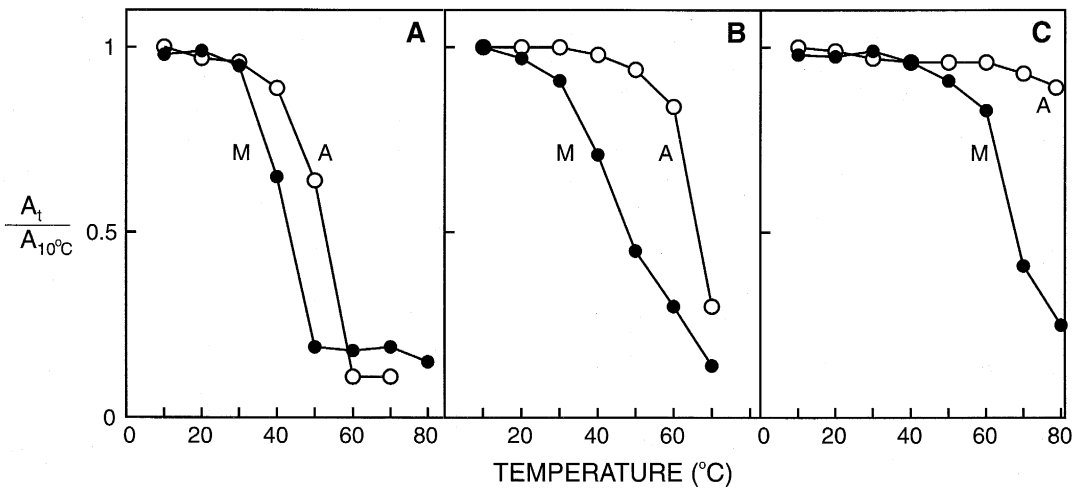

Fig. 2. Effect of temperature on the visible absorption at A_{max} of biliprotein monomers and aggregates. A: phycocyanin 645. (*Chroomonas* sp.) B: C-phycocyanin (*P. luridum*); C: C-phycocyanin (SyI). Monomer curves are designated M and aggregates are labelled A.

near UV coupled with a loss of visible absorption demonstrates that the bilins have become cyclic. The bilins are linear in the ordered protein but become cyclic when the protein becomes strongly disordered. For aggregated protein, the bilins remained linear at 70°C. For monomers, some bilin cyclization is observed at 60°C and by 70°C major changes have occurred in bilin conformation. Clearly, monomers have become disordered at 70°C and could not further associate to form trimers and ultimately phycobilisomes. It is reasonable, therefore, to propose that either of two possible events could protect the monomers from thermal denaturation. The monomers may, before they can denature, aggregate very quickly to trimers, which are stable at 70°C. A second possibility is that some cellular factor might interact with monomers and offer them additional thermal stabilization.

Future work on the stability of the separated α and β polypeptides both with and without bilins would also be of interest.

3.3. Phycobilisomes / C-phycocyanin

C-Phycocyanin (SyI) differed in two ways from other C-phycocyanins: it had a blue-shifted bilin absorption maximum and a wider range of temperature stability. Although the absorption maximum at 70°C was unique (Fig. 1A), the fluorescence emission of C-phycocyanin (SyI) at 70°C was found at the normal wavelength maximum (data not shown). These results suggested that the bilin-to-bilin energy transfer from C-phycocyanin to allophycocyanin was the same for SyI as for other biliproteins; Förster energy transfer from C-phycocyanin (SyI) to allophycocyanin (SyI) depends on the overlap between donor emission and acceptor absorption. These observations suggest a

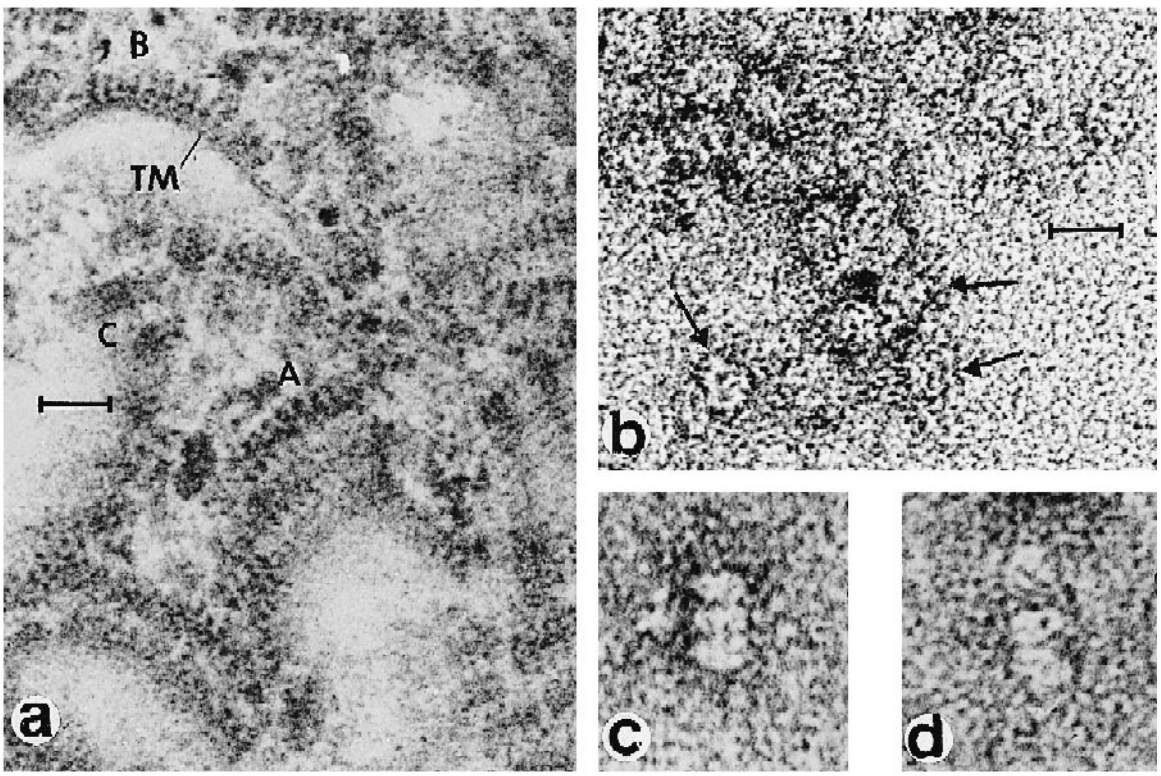

Fig. 4. Electron microscopy of *S. lividus* (SyI). A thin section (a) and negative stained oligomers of C-phycocyanin (b–d). Three different views of phycobilisomes are shown in panel (a): a grazing section over a row of phycobilisomes (A), phycobilisomes attached to the thylakoid membrane (TM) (B), and a region of cross-section through the phycobilisomes (C). Negatively stained preparations revealed both the top views (b, arrows) and side views (c) of the rods from phycobilisomes. In panel d, two hexamers appear in side view, with a third appearing as an oblique frontal view. Magnification bars in panel a: 55 nm, in panels b–d: 15 nm.

similar function for the protein, but is the structure of this SyI protein similar to those of other biliproteins?

Biliproteins are arrayed along the thylakoids of cyanobacteria as phycobilisomes [2,23–25]. Three views from thin sections of *S. lividus* show typical phycobilisomes (Fig. 4a). Purified C-phycocyanin of SyI should exist as rods and breakdown products of rod dissociation. Electron micrographs of negatively stained C-phycocyanin showed some particles that resembled the rods of phycobilisomes (Fig. 4b–d). The results show both top (Fig. 4b) and side views (Fig. 4c). The rods are seen as stacks of three hexameric aggregates (Fig. 4c). The electron micrograph (Fig. 4b) showing structures with a central object could be C-phycocyanin hexamers viewed from the top with a centrally located linker polypeptide. In Fig. 4d, there is either a trimeric stack of disks, or a two-hexamer stack with a randomly positioned hexamer nearby. These electron microscopy results resemble those from mesophilic organisms [4].

In the phycobilisomes, C-phycocyanin will generally be found as stacks of two or more disks, and each disk is an $\alpha_6\beta_6$ assembly of the α and β polypeptides, and the disks are connected by linker polypeptides. When the phycobilisomes from mesophilic algae are extracted into dilute phosphate buffer, the rods detach from the allophycocyanin core and the rods dissociate into $\alpha_6\beta_6$, $\alpha_3\beta_3$, $\alpha\beta$ and $(\alpha_6\beta_6)_2$ units. However, the dissociation of SyI C-phycocyanin is very limited and the association may, under certain conditions, be retained as intact rods, $(\alpha_6\beta_6)_3$ plus linkers [10]. It was of interest to determine whether the rods from SyI C-phycocyanin would dissociate to $\alpha_6\beta_6$, $\alpha_3\beta_3$, and $\alpha\beta$ as do other C-phycocyanins. The purified SyI C-phycocyanin was, therefore, treated with 1 or 2 M NaSCN, a known biliprotein dissociating agent [4]. After the NaSCN was dialyzed away, the protein was analyzed by gel-filtration column chromatography. The eluted profiles (data not shown) showed molecular masses indicating that the protein was a mixture of $\alpha\beta$ and $\alpha_3\beta_3$ [10], which is in agreement with results found for mesophilic C-phycocyanin. The absence of $(\alpha_6\beta_6)_n$ indicates loss of linker polypeptides.

In order to compare C-phycocyanin from *S. lividus* (SyI) with other C-phycocyanins, its individual subunits were examined. The C-phycocyanin was fully dissociated and analyzed by electrospray mass spectrometry. Other C-phycocyanins have been shown to have two subunits with molecular masses between 16 and 20 kDa [26–30]. The smaller is named α and the slightly larger β. The β subunit has two chromophores and α has one, and the β subunit has several more amino acids that α. The electrospray results showed this C-phycocyanin to have strong homology to the other with α at 18 466–18 483 Da and β at 19 627 Da (data not shown). It was concluded from these data that phycobilisomes, C-phycocyanin dissociation products, and C-phycocyanin subunits are the same for this alga and many other known cyanobacteria. Previous studies [10] demonstrated that C-phycocyanin (SyI) has the same bilin content as other C-phycocyanins.

3.4. C-phycocyanin aggregation

Another thermophilic strain, *S. lividus* (SyIII) has rods that dissociate extensively at 20°C to $\alpha_6\beta_6$ and $\alpha_3\beta_3$ in pH 6.0, 0.1 ionic strength, buffer [9,22]. Moreover, this other thermophilic C-phycocyanin appears to dissociate more completely than mesophilic C-phycocyanins under the same conditions. This creates a situation where SyI protein is 'temperature-resistant' and retains a high assembly level [10] and functionality (Fig. 1) over a wide temperature range, and C-phycocyanin SyIII dissociates at lower temperatures, a 'cold-dissociated' protein. *S. lividus* (SyIII) grows up to 55°C. We, therefore, examined its C-phycocyanin at 20°C and 50°C by absorption spectroscopy to determine if it was disordered at 20°C as well as dissociated. The absorption spectra for 300–700 nm behaved with temperature exactly like C-phycocyanin from the SyI strain (Fig. 1A). The absorption in the near UV was identical at 20°C and 50°C, and the visible band was very slightly attenuated at 50°C and reversed readily when returned to 20°C (data not shown). The SyIII C-phycocyanin was cold-dissociated, but not disordered at low temperature. This SyIII case is somewhat analogous to cold denaturation where some proteins and polypeptides undergo a conformation change at low temperatures to a more disordered state ([11] and references therein), but for SyIII C-phycocyanin only dissociation and not denaturation is involved.

The mechanism for this cold-dissociation could be uncoupling or partial uncoupling between the C-

phycocyanin and the linker polypeptides, since linkers are responsible for the disk-to-disk biliprotein assembly. Berns and Scott [22] discovered 'cold-dissociation' for SyIII C-phycocyanin, and found that bringing the temperature up to 49°C greatly enhanced the aggregation.

Many of the studies comparing C-phycocyanins from mesophilic and thermophilic algae were done using solutions wherein the states of aggregation were different between solutions under comparison. This situation was difficult to avoid because the C-phycocyanins from thermophiles and mesophiles possess different aggregation (Fig. 2), but the various aggregates may differ in thermal stabilization. Moreover, the opportunity now exists to make a mesophile-thermophile comparison for two C-phycocyanins both as monomers. Although monomers are less stable then their corresponding aggregates, the mesophilic monomers (Fig. 2B) are clearly much less temperature stable than thermophilic C-phycocyanin monomers (Fig. 2C). In addition, previous temperature studies may have involved C-phycocyanin aggregates that included linker polypeptides. Monomers are totally devoid of linkers, and it is clear that C-phycocyanin itself is more stable when obtained from the thermophilic source (Fig. 2). The results of x-ray crystallographic studies of mesophilic and thermophilic biliproteins from cyanobacteria and red algae have revealed that they are all very similar in secondary and tertiary structures [31–34]. The intrinsic stability of the thermophilic C-phycocyanin must reside in the individual interactions of some of the amino acids that compose these structural elements. The factors in how proteins achieve stability at very high temperatures have been reviewed [35].

Acknowledgements

The mass spectrometer was funded by NSF Grant BIR-92-17648. The electron microscopy was done using the Wadsworth Center's Electron Microscopy Core Facility. We thank Dr. William A. Samsonoff for help and advice. The Wadsworth Center's Biochemistry Core was utilized in these studies.

References

[1] D.S. Berns, R. MacColl, Chem. Rev. 89 (1989) 807–825.
[2] E. Gantt, in: M. Levandowsky, S.A. Hutner (Eds.), Biochemistry and Physiology of Protozoa, vol. 1, 2nd edn., Academic Press, New York, 1979, p. 121.
[3] A.R. Holzwarth, Physiol. Plant 83 (1991) 518–528.
[4] R. MacColl, D. Guard-Friar, Phycobiliproteins, CRC Press, Boca Raton, FL, 1987.
[5] E. Moerschel, Photosynthesis 25 (1991) 137–144.
[6] H. Scheer, Angew. Chem. Int. Ed. (1981) 241–261.
[7] H. Zuber, in: J. Barber (Ed.), The Light Reactions, Elsevier Science, Amsterdam, 1987, pp. 197–259.
[8] R. MacColl, M.R. Edwards, C. Haaksma, Biophys. Chem. 8 (1978) 369–376.
[9] R. MacColl, M.R. Edwards, M.H. Mulks, D.S. Berns, Biochem. J. 141 (1974) 419–425.
[10] M.R. Edwards, R. MacColl, L.E. Eisele, Biochim. Biophys. Acta 1276 (1996) 64–70.
[11] E. Lacassie, A. Delmas, C. Meumier, Y. Trudelle, Int. J. Peptide Protein Res. 48 (1996) 249–258.
[12] R. MacColl, D.S. Berns, N.L. Koven, Arch. Biochem. Biophys. 146 (1971) 477–482.
[13] R. MacColl, H. Malak, J. Cipollo, B. Label, G. Ricci, D. MacColl, L.E. Eisele, J. Biol. Chem. 270 (1995) 27555–27561.
[14] N. Sreerama, R.W. Woody, Anal. Biochem. 209 (1993) 32–44.
[15] M.P. Debreczeny, K. Sauer, J. Zhou, D.A. Bryant, J. Phys. Chem. 99 (1995) 8420–8431.
[16] R. MacColl, S. Kapoor, D.R. Montelesse, S. Kukadia, L.E. Eisele, Biochemistry 35 (1996) 15436–15439.
[17] C.-H. Chen, L.G. Roth, R. MacColl, D.S. Berns, Biophys. Chem. 50 (1994) 313–321.
[18] L.G. Roth, D.S. Berns, C.-H. Chen, Biophys. Chem. 60 (1996) 89–97.
[19] C.-H. Chen, D.S. Berns, Biophys. Chem. 8 (1978) 203–213.
[20] C.-H. Chen, D.S. Berns, Plant Physiol. 66 (1980) 596–599.
[21] H.W.O. Kao, M.R. Edwards, D.S. Berns, Biochem. J. 147 (1975) 63–70.
[22] D.S. Berns, E. Scott, Biochemistry 5 (1966) 1528–1533.
[23] M.R. Edwards, E. Gantt, J. Cell Biol. 50 (1971) 896–900.
[24] E. Gantt, Annu. Rev. Plant Physiol. 32 (1981) 327–347.
[25] K. Ohki, E. Gantt, C.A. Lipschultz, M.C. Ernst, Plant Physiol. 79 (1985) 943–948.
[26] G. Frank, W. Sidler, H. Widmer, H. Zuber, Hope-Seyler's Z. Physiol. Chem. 358 (1978) 1491–1507.
[27] P. Füglistaller, F. Suter, H. Zuber, Hoppe-Seyler's Z. Physiol. Chem. 364 (1983) 691–712.
[28] G.D. Offner, A.S. Brown-Mason, M.M. Ehrhardt, R.F. Troxler, J. Biol. Chem. 256 (1981) 12167–12175.
[29] R.F. Troxler, M.M. Ehrhardt, A.S. Brown-Mason, G.D. Offner, J. Biol. Chem. 256 (1981) 12176–12184.
[30] H. Zuber, in: J. Barber (Ed.), The Light Reactions, Elsevier Science, Amsterdam, 1987, p. 197.

[31] M. Duerring, G.B. Schmidt, R. Huber, J. Mol. Biol. 217 (1991) 577–592.
[32] T. Schirmer, W. Bode, R. Huber, J. Mol. Biol. 196 (1987) 677–695.
[33] T. Schirmer, W. Bode, R. Huber, W. Sidler, H. Zuber, J. Mol. Biol. 184 (1985) 257–277.
[34] T. Schirmer, R. Huber, M. Schneider, W. Bode, M. Miller, M.L. Hackert, J. Mol. Biol. 188 (1986) 651–676.
[35] R. Jaenicke, H. Schurig, N. Beaucamp, R. Ostendorp, in: F.M. Richards, D.S. Eisenberg, P.S. Kim (Eds.), Advances in Protein Chemistry, Academic Press, San Diego, 1966, p. 181.