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COMPARISON OF THE STABILITY OF PHYCOCYANINS FROM THERMOPHILIC, MESOPHILIC, PSYCHROPHILIC AND HALOPHILIC ALGAE

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Protein unfolding of eight different phycocyanins was investigated utilizing circular dichroism and visible spectra. The phycocyanin samples were extracted from algae that are normally found in vastly different environments, and are classified as mesophilic, thermophilic, halophilic and psychrophilic. The ability of these proteins to resist the denaturant urea is in the order of thermophile > mesophile, halophile > psychrophile. Based on a two-state approximation the apparent free energies of protein unfolding at zero urea denaturant concentration, $\Delta G_{app}^{H_2O}$, were found to range from 2.4 to 8.8 kcal/mole for the eight phycocyanins at pH 6 and 25°C. The proteins from the thermophile are generally more stable than those from the mesophile. An extra stability of the halophile is believed due to the specific interaction of the proteins and the ions in solution. A correction for $\Delta G_{app}^{H_2O}$ due to minor amino acid differences reveals that the stability and the structural properties of these proteins are primarily affected by this minor difference in amino acid compositions.

1. Introduction

The biliprotein, phycocyanin, is found in blue-green and red algae. It is both an energy-transfer pigment in Photosystem II [1], and a modifier of the electron flow across chloroplast extract bilayer membranes [2]. Enzymatic activity in the conventional sense is not present in this protein. The protein is found in vivo in an aggregated state in structures called phycobilisomes [3]. Cyanophyta (blue-green algae) are among the oldest known organisms [4] and are found to exist in a diversity of ecological niches and consequently thrive under vastly different environmental stress. This characteristic leads to the use of phycocyanin as a probe in the studies of the effect of variations in environmental conditions on the structure and function of proteins. The characterization of phycocyanin extracted from different microorganisms such as *Mastigocladus laminosus* (I-30-m), *Mastigocladus laminosus* (NZ-DB2-m), *Cyanidium caldarium, Anabaena variabilis, Plectonema calothricoides, Phormidium luridum, Microcoleus vaginatus,* and *Coccochloris elabens* has been reported [5–9]. These organisms were grown in the laboratory under the specific culture conditions that reflect the environmental niche in which they are normally encountered. The above mentioned algae are classified as thermophilic (high temperature), mesophilic (room temperature), psychrophilic (low temperature) or halophilic (high salt) alga according to the temperature or the salt concentration of their culture media. The amino acid analyses, partial amino acid sequences and immunochemical studies of phycocyanin from this diverse group of organisms demonstrate that these phycocyanins have a striking degree of similarity [5–9], even though in their natural environment they are exposed to markedly different temperature and salt conditions. It is suggested that the investigation of these algal proteins is useful in the understanding of the subtleties of protein structure that are factors in the organisms' ability to survive environmental stresses [5–9].

The protein aggregation property of phycocyanin results from both protein-protein and protein-solvent interactions. At 25°C, the purified phycocyanins in a concentration of about 10 mg/ml contain a mixture of principally three different aggregates, 6S (trimer), 11S (hexamer), and 19S (dodecamer), in pH 6 or pH 7, I (ionic strength) 0.1 sodium phosphate buffer. It is possible to isolate phycocyanins under conditions

where there will be large amounts of aggregates larger than 11S. The relative amounts of the several aggregates found in protein prepared from different algal sources can vary widely [5, 7–9]. Hydrophobic interactions are believed to play an important role in the aggregation of these proteins [10]. The effects of small molecules such as tetraalkylammonium salts, phenol and naphthols on their aggregation properties at room temperature have also been studied. It was found that the changes in the aggregation state caused by phenol and naphthols differ between proteins isolated from mesophile and thermophile [5, 10].

The thermostability of enzyme activity from some thermophilic and mesophilic organisms have been investigated [11–13]. It has been shown that the proteins such as cytoplasmic proteins, bacterial flagella and triosephosphate isomerase from thermophiles are more thermostable and more resistant to denaturants than those from the mesophile [12, 13]. There are few studies of thermal or perturbant denaturation of the proteins from a psychrophilic source compared with a thermophile or a mesophile. One recent report is that triosephosphate isomerase from a psychrophile was found to have less resistance to heat and urea denaturation than that from the thermophile or the mesophile [14]. The effect of salt on the activity, and stability of some enzymes from halophilic organisms has also been studied. Enzymes from extremely halophilic bacteria have been shown to require high concentrations of salt for both activity and stability [15–17].

The native conformation of a globular protein is about 5–10 kcal/mole more stable than a randomly coiled conformation [18]. By studying the protein denaturation in urea or guanidine hydrochloride solution, one can obtain the apparent free energy of protein unfolding at zero denaturant concentration $\Delta G^{H_2O}_{app}$ [19, 20] which is an estimate of the native conformation stability relative to the coiled form. In the investigation of the denaturation of phycocyanin by urea, we have recently reported $\Delta G^{H_2O}_{app}$ for phycocyanin from *P. luridum* [21]. There is, however, no study that reports simultaneous investigations that compare the structural stability of the same protein extracted from organisms which are grown in four different environments, high, room and low temperatures and high salt concentration. The present investigation is a study of the denaturation of a broad spectrum of phycocyanins extracted from organisms that are grown in these four different environments, and presents and compares the free energies of unfolding and the stability of these phycocyanins to see if any correlation may be made between the estimated relative stability of these proteins and their structural properties.

2. Materials and methods

Seven blue-green algae, *M. laminosus* (I-30-m), *M. laminosus* (NZ-DB2-m), *C. caldarium*, *A. variabilis*, *P. calothricoides*, *M. vaginatus* and *C. elabens*, were cultured under specific conditions. After harvest the cells were lysed by lysozyme. The extraction and purification of the phycocyanins was described previously [10]. The protein solutions were repeatedly precipitated with 50% ammonium sulfate, and the protein dissolved in pH 6.0 phosphate buffer was dialyzed in 35% ammonium sulfate solution and fractionated several times until the ratio of A_{620} to A_{280} was 4 or greater. Sodium dodecyl sulfate acrylamide gel electrophoresis has been performed on all proteins and the only protein present in the purified preparation is phycocyanin. The purified proteins were stored in 50% saturated ammonium sulfate solution in a 4°C cold room.

Urea (ultra pure grade from Schwarz/Mann Co.) was purified by treatment with a mixed ion exchange resin (AG-501-X8 analytical grade, Bio-Rad Laboratories) as described previously [21].

Urea solutions from 1 M to 9 M were prepared by dissolving appropriate amounts of urea in 2 ml volumetric flasks containing pH 6, $I = 0.1$ buffer. The addition of urea solutions resulted in only a small change in the pH of the solution (pH = 6.3 at 7 M urea as compared to pH = 6.0 at zero urea). Since it was necessary to refer to the same reference state, it was decided not to add additional acid to the solutions and consequently the small change in the pH of the protein solutions was not readjusted after urea addition. About 10–20 μl of phycocyanins were added to the urea solutions to give an optical density (OD) of the solution of approximately 0.8–0.9 at 620 nm. The mixed protein-urea solutions were kept in 4°C cold room overnight to make sure the denaturation process was completed. It was found that

in the presence of urea the absorbance at 615 nm of phycocyanin extracted from *Nostoc punctiformis* decreases as time increases and then levels off [22]. The present absorption and circular dichroism (CD) measurements were performed on urea-protein solutions that were monitored over a sufficient period of time to ascertain no additional changes were occurring. In order to make proper comparison of the temperature-dependent thermodynamic quantity, $\Delta G_{app}^{H_2O}$ and to correlate this quantity with the known aggregation data at room temperature, all measurements regardless of the sources of phycocyanins were carried out at the same temperature, 25°C.

Visible absorption spectra and CD of phycocyanin in the presence and absence of urea were measured with a Cary model 14 spectrophotometer and a Cary model 61 CD spectropolarimeter, respectively. To obtain accurate ellipticity data, a constantly low scanning rate was used during the measurements. The CD cells had a 0.1 cm light path. The concentrations of proteins were determined on the basis of a specific extinction coefficient of 6.0 at 620 nm for a 1 mg/ml solution of protein in pH 6 buffer [10]. Phycocyanins have a maximum absorption at approximately 620 nm in the visible range. The absorption spectra were measured as a function of urea concentration in the ranges from 710 to 450 nm. The CD spectra of phycocyanin showed a strong positive band at around 630 nm and a strong negative band at 330 nm [21, 23]. In the present investigation, we were interested in the CD spectral region in which it is generally accepted manifestations occur of changes in protein conformation [24]. The CD spectra of seven phycocyanins were therefore studied from 280 to 200 nm.

3. Results

The dependence of the molar ellipticities of CD spectra at 222 nm, $[\theta]_{222}$ (deg cm^2/decimole), of

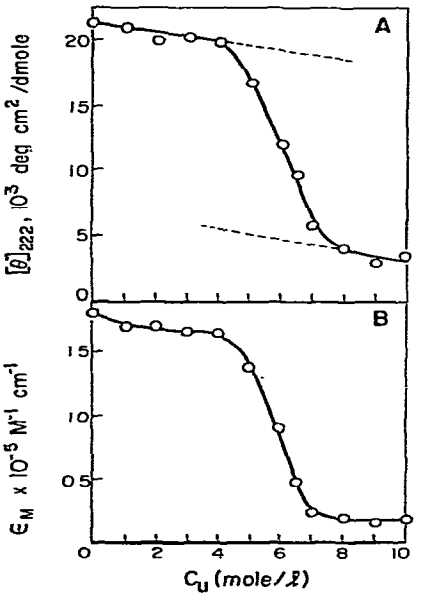

Fig. 1. (a) CD spectrum of phycocyanin from *M. laminosus* (37°C, I-30-m) as a function of urea concentration. Protein concentration = 0.12 mg/ml. (b) Molar extinction coefficient (ϵ_m) at the visible absorption maximum of phycocyanin from *M. laminosus* (37°C, I-30-m) as a function of urea concentration (C_u). Protein concentration = 0.12 mg/ml.

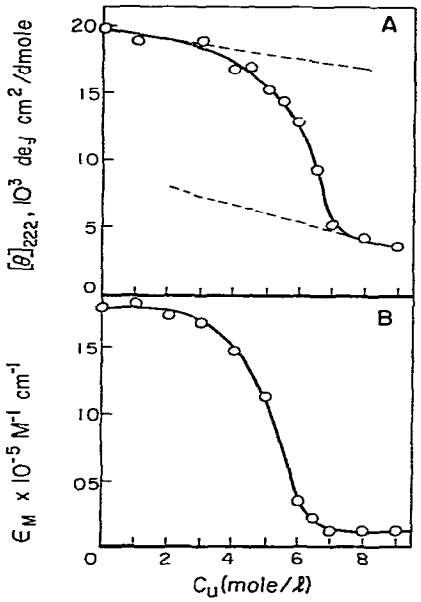

Fig. 2. (a) CD spectrum of phycocyanin from *M. laminosus* (50°C, NZ-DB2-m) as a function of urea concentration. Protein concentration = 0.11 mg/ml. (b) Molar extinction coefficient (ϵ_m) at the visible absorption maximum of phycocyanin from *M. laminosus* (50°C, NZ-DB2-m) as a function of urea concentration (C_u). Protein concentration = 0.11 mg/ml.

seven phycocyanins on the concentration of urea (C_u) is plotted in figs. 1a–7a. Each of these plots consists of three regions representing the protein before unfolding, the protein undergoing unfolding and the protein after unfolding, respectively.

The visible spectra of phycocyanins show a maximum at 615–620 nm. In the presence of 1–9 M urea, the OD at the maximum absorption decreases as the concentration of urea in the protein solution increases. The molecular weight of phycocyanin monomer, 3×10^4 dalton, was used to calculate the molar extinction coefficients (ϵ_M). Plots of ϵ_M at the visible absorption maximum of the phycocyanins as a function of C_u are presented in figs. 1b–7b.

A two-state mechanism has been used to explain the denaturation of phycocyanin from *P. luridum* [21]. This assumption is also adopted in the present studies for phycocyanins from other sources. The equilibrium constant (K) can be determined from the CD data by using the following equation:

$$\text{Native state} \rightarrow \text{Denatured state} \tag{1}$$

$$K = \frac{[\theta]_N - [\theta]}{[\theta] - [\theta]_D}, \tag{2}$$

where $[\theta]$ denotes the observed molar ellipticity at 222 nm for protein in urea solution C_u and $[\theta]_N$ and $[\theta]_D$ are the molar ellipticities which the native and the completely unfolded proteins would have under the same conditions. The magnitudes of $[\theta]_N$ and $[\theta]_D$ at C_u can be obtained from the extrapolated straight lines as shown in figs. 1a–7a. The apparent free energy of denaturation, ΔG_{app}, can then be calculated according to

$$\Delta G_{app} = -RT \ln K. \tag{3}$$

Values of K and ΔG_{app} obtained from the analysis of the data shown in figs. 1a–7a are presented in table 1.

In the studies of denaturation of ribonuclease, lysozyme, α-chymotrypsin and β-lactoglobulin by urea and guanidine hydrochloride, Greene and Pace

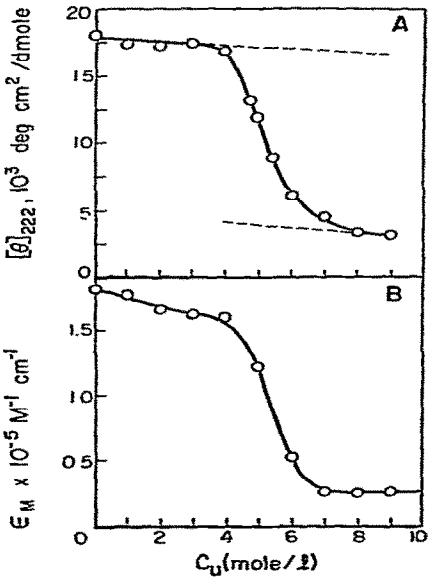

Fig. 3. (a) CD spectrum of phycocyanin from *C. caldarium* as a function of urea concentration. Protein concentration = 0.15 mg/ml. (b) Molar extinction coefficient (ϵ_m) at the visible absorption maximum of phycocyanin from *C. caldarium* as a function of urea concentration (C_u). Protein concentration = 0.15 mg/ml.

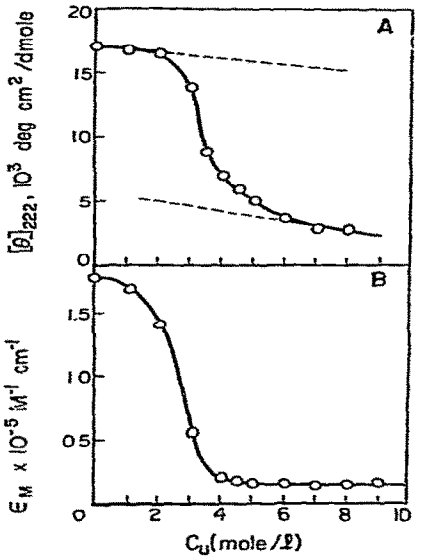

Fig. 4. (a) CD spectrum of phycocyanin from *A. variabilis* as a function of urea concentration. Protein concentration = 0.16 mg/ml. (b) Molar extinction coefficient (ϵ_m) at the visible absorption maximum of phycocyanin from *A. variabilis* as a function of urea concentration (C_u). Protein concentration = 0.16 mg/ml.

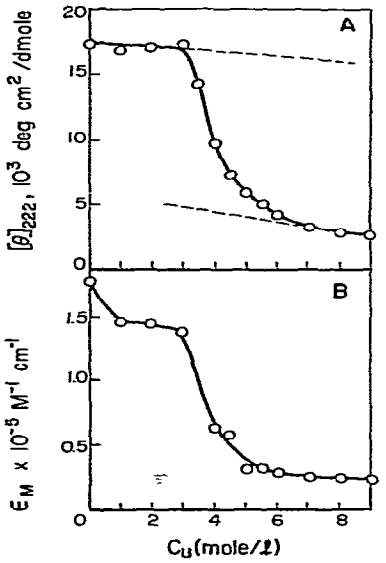

Fig. 5. (a) CD spectrum of phycocyanin from *P. calothricoides* as a function of urea concentration. Protein concentration = 0.15 mg/ml. (b) Molar extinction coefficient (ϵ_m) at the visible absorption maximum of phycocyanin from *P. calothricoides* as a function of urea concentration. Protein concentration = 0.15 mg/ml.

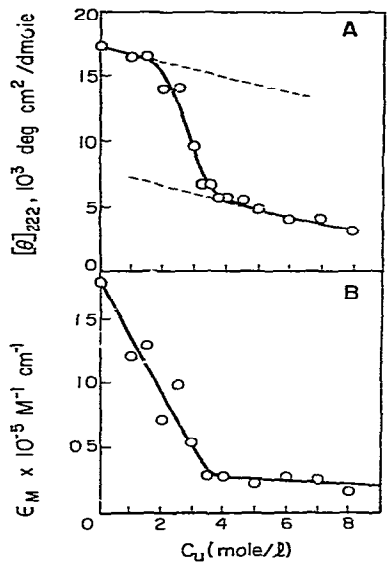

Fig. 6. (a) CD spectrum of phycocyanin from psychrophilic *M. vaginatus* as a function of urea concentration. Protein concentration = 0.12 mg/ml. (b) Molar extinction coefficient (ϵ_m) at the visible absorption maximum of phycocyanin from psychrophilic *M. vaginatus* as a function of urea concentration (C_u). Protein concentration = 0.13 mg/ml.

[19, 20] obtained $\Delta G_{app}^{H_2O}$, the apparent free energy of unfolding at zero denaturant concentration, by using a least squares analysis to fit ΔG_{app} to the equation

$$\Delta G_{app} = \Delta G_{app}^{H_2O} - mC_u. \quad (4)$$

This method is also adopted here. The least square coefficients m and the calculated $\Delta G_{app}^{H_2O}$ for eight phycocyanins are summarized in table 2. The culture condition of each alga from which the protein was attained and the urea concentration at which the protein denaturation is half way through, $(C_u)_{1/2}$, are also included in the table.

The amino acid compositions of the eight phycocyanins are similar [4–7]. An arbitrarily composite idealized amino acid composition based on a 30,000 molecular weight monomer is used as a reference value in table 3 and deviations from this are catalogued. In order to make a relevant comparison of the structure of phycocyanins by utilizing $\Delta G_{app}^{H_2O}$, one needs to employ $\Delta G_{app}^{H_2O}$ values that take into account on any difference in the amino acid composition from the reference value. A correction for $\Delta G_{app}^{H_2O}$ due to the amino acid composition differences can be carried out on the basis of

$$\Delta G_{app(c)}^{H_2O} = \Delta G_{app}^{H_2O} + \sum_i (\delta n_i)(\Delta F_{t(i)}), \quad (5)$$

where $\Delta G_{app(c)}^{H_2C}$ is the apparent free energy of protein unfolding at zero denaturant concentration corrected for differences in amino acid composition, δn_i is the deviation in the number of amino acid residue i and $\Delta F_{t(i)}$ is the free energy of transfer for group i from one solvent composition to another. Free energies of transfer $\Delta F_{t(i)}$ from water to aqueous urea solutions at 25°C are taken from Nozaki and Tanford [25]. It is assumed that isoleucine and leucine have the same $\Delta F_{t(i)}$ [26]. The calculated $\delta n_i \Delta F_{t(i)}$ are shown in table 3 and values of $\Delta G_{app(c)}^{H_2O}$ are presented in table 2.

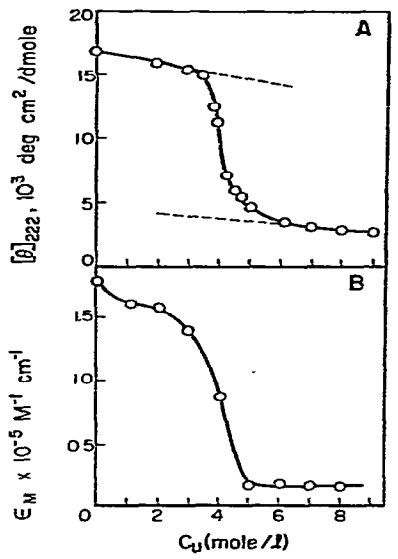

Fig. 7. (a) CD spectrum of phycocyanin from *C. elabens* as a function of urea concentration. Protein concentration = 0.14 mg/ml. (b) Molar extinction coefficient (ϵ_m) at the visible absorption maximum of phycocyanin from *C. elabens* as a function of urea concentration. Protein concentration = 0.14 mg/ml.

4. Discussion

A comparison of CD and visible absorption spectra as shown in figs. 1–7 reveals that the shapes of the denaturation curves are quite similar for both CD and absorption spectra. In addition, there is good agreement in the denaturant concentration for which the protein undergoes complete denaturation.

Table 2 shows that, in general, the magnitudes of the denaturant concentration at the midpoint of the denaturation curve, $(C_u)_{1/2}$, and thus the ability of phycocyanin to resist the denaturant urea is in the order of thermophile > mesophile, halophile > psychrophile. It should be noted here that the halophile is grown at room temperature as are the mesophiles although studies in laboratory cultivation indicate that the halophilic organism will grow at temperatures as high as 45°C [7]. In the examination of the effects of temperature and urea on the enzyme denaturation, the purified thermophilic, mesophilic and psychrophilic triosephosphate isomerases show great differences in susceptibility to denaturants [15]. These enzymes lose 50% of the initial activity after 10 min of incubation at 28°C for psychrophile, 45°C for mesophile, and 64°C for thermophile. The resistance of these three proteins to urea denaturation was found to follow their resistances to heat denaturation. The present results agree with the observations that a protein from a thermophilic source seems somewhat more impervious than the mesophilic one to reagents which are thought to disrupt hydrophobic interactions [13, 27, 28]. The thermophilic protein is considered to have a more rigid structure which is less easily denatured by the change of an external parameter such as temperature and the nature of the solvent [27, 29]. This conclusion is supported by the observation that 10^{-2} M phenol and 10^{-3} M naphthols promote the aggregation of phycocyanin from mesophilic *P. luridum* by increasing its hexamer concentration and reducing the amount of trimer [10], but phenol and naphthol have no effect on phycocyanins isolated from the two cultures of *M. laminosus* [5].

The magnitude of $\Delta G_{app}^{H_2O}$ for phycocyanin derived from algae subject to four different degrees of environmental stress is compared in table 2 and the values are in the order of halophile > thermophile, psychrophile > mesophile. The observation that the ranking of the order of $\Delta G_{app}^{H_2O}$ does not coincide with that of $(C_u)_{1/2}$ does not indicate a contradiction between $\Delta G_{app}^{H_2O}$ and $(C_u)_{1/2}$ data, since these quantities do not have a simple direct correlation. For instance, the values of $(C_u)_{1/2}$ are 6.96, 5.21, 4.04, and 5.01 M for ribonuclease, lysozyme, α-chymotrypsin, and β-lactoglobulin, respectively, while those of $\Delta G_{app}^{H_2O}$ are 7.7, 5.8, 8.4 and 10.5 kcal/mole, respectively [20]. In general, the present data demonstrate that the average $\Delta G_{app}^{H_2O}$ for thermophile and psychrophile are about 1–2 kcal/mole higher than the value for the mesophile, while $\Delta G_{app}^{H_2O}$ of the halophile is about 4 kcal/mole higher than the average $\Delta G_{app}^{H_2O}$ of thermophile or psychrophile. The extra stability of a protein from a thermophile has been found for D-glyceraldehyde-3-phosphate dehydrogenase from a thermophilic bacterium in which an increase of about 40°C in the melting point due to a stabilization energy of 10 kcal/mole was reported [30]. There is an absence of data in the literature on the relative stability of the same protein isolated from psychrophile and mesophile. The reason

Table 1
K and ΔG_{app} values of phycocyanins calculated in the process of urea denaturation

C_u mole/l	*M. laminosus* (37°C)		*M. laminosus* (50°C)		*C. caldarium*		*A. variabilis*		*P. calothricoides*		*M. vaginatus*		*C. elabens*	
	K	ΔG_{app}	K	ΔG_{app}	K	ΔG_{app}	K	ΔG_{app}	K	ΔG_{app}	K	ΔG_{app}	K	ΔG_{app}
2.15											0.118	1.27		
2.30											0.250	0.82		
2.50											0.484	0.43		
2.70											0.863	0.09		
2.90											1.436	−0.21		
3.10											2.481	−0.54		
3.30							0.966	0.02			4.875	−0.94		
3.50							1.600	−0.28						
3.75							2.343	−0.50						
3.80									0.746	0.17				
3.85													0.299	0.82
4.00							3.179	−0.68	1.296	−0.15			0.514	0.39
4.10													1.036	−0.02
4.25							4.318	−0.87	2.024	−0.42			2.200	−0.47
4.50			0.160	1.09	0.236	0.86	5.500	−1.01	3.133	−0.68			3.870	−0.80
4.75	0.148	1.13	0.206	0.94	0.440	0.49	6.800	−1.14	4.208	−0.85			6.400	−1.10
5.00	0.248	0.83	0.258	0.80	0.658	0.25	9.727	−1.35	5.579	−1.02				
5.25	0.352	0.62	0.326	0.66	1.220	−0.12	13.75	−1.55	8.000	−1.23				
5.50	0.515	0.39	0.404	0.54	1.848	−0.36	18.83	−1.74	10.45	−1.39				
5.75	0.750	0.17	0.526	0.38	2.743	−0.60			14.88	−1.60				
6.00	1.042	−0.02	0.725	0.19	4.038	−0.83			20.17	−1.78				
6.25	1.492	−0.24	1.069	−0.04	5.895	−1.05								

Table 2
Apparent free energy of unfolding of phycocyanins

Source of phycocyanin	Culture condition of organism	$(C_u)_{1/2}$ mole/l	$\Delta G^{H_2O}_{app}$ kcal/mole	m	$\sum_i (\delta n_i)(\Delta F_{t(i)})$ [a] kcal/mole	$\Delta G^{H_2O}_{app(c)}$ [b] kcal/mole
Thermophilic						
M. laminosus (I-30-m)	37°C, pH 7	6.0 ± 0.2	5.3 ± 0.1	0.89 ± 0.02	−0.2 ± 0.02	5.1 ± 0.1
M. laminosus (NZ-DB2-m)	50°C, pH 7	6.2 ± 0.2	3.9 ± 0.1	0.62 ± 0.02	0.3 ± 0.03	4.2 ± 0.2
C. caldarium	50–55°C, pH 2	5.2 ± 0.2	5.3 ± 0.2	1.02 ± 0.03	−0.6 ± 0.06	4.7 ± 0.3
Mesophilic						
A. variabilis	25°C, pH 7	3.3 ± 0.2	2.4 ± 0.1	0.74 ± 0.03	0.9 ± 0.09	3.3 ± 0.2
P. calothricoides	25°C, pH 7	3.9 ± 0.2	3.2 ± 0.2	0.84 ± 0.03	1.6 ± 0.16	4.8 ± 0.3
P. luridum [c]	25°C, pH 7	5.0 ± 0.2	4.2 ± 0.3	0.83 ± 0.05	0.7 ± 0.07	4.9 ± 0.5
Psychrophilic						
M. vaginatus	4°C, pH 7	2.8 ± 0.2	5.1 ± 0.2	1.83 ± 0.07	−0.4 ± 0.04	4.7 ± 0.2
Halophilic						
C. elabens	26°C, 15–20% NaCl	4.1 ± 0.2	8.8 ± 1.1	2.12 ± 0.26	1.0 ± 0.1	9.8 ± 1.2

[a] No standard deviation was reported in ref. [25] for $\Delta F_{t(i)}$. It is assumed to be ± 10% here.
[b] See eq. (5) for definition.
[c] Recalculated from ref. [21].

Table 3
Amino acid compositions and calculation of $(\delta n_i)(\Delta F_{t(i)})$ of the eight phycocyanins [a]

Residues	Reference No. of amino acid residues [b]	Deviant amino acid residue content	δn_i [c]	$\Delta F_{t(i)}$ [d] cal/mole	$(\delta n_i)(\Delta F_{t(i)})$ kcal/mole
(A) Polar					
Acidic					
Asp	30				
Glu	26	19 (PC) 18 (PL) 29 (CE)	−7 (PC) −8 (PL) 3 (CE)	−120 (PC) −140 (PL) −124 (CE)	0.8 (PC) 1.1 (PL) −0.4 (CE)
Basic					
Lys [e]	11	9 (CE)	−2 (CE)	–	–
His	2				
Arg	17				
Hydroxy					
Thr	16	19 (MV) 13 (PL)	3 (MV) −3 (PL)	−48 (MV) −58 (PL)	−0.1 (MV) 0.2 (PL)
Ser [f]	18	22 (MV) 14 (CE)	4 (MV) −4 (CE)	0 (MV) 0 (CE)	0 (MV) 0 (CE)

Table 3 (continued)

Residues	Reference No. of amino acid residues [b]	Deviant amino acid residue content	δn_i [c]	$\Delta F_{t(i)}$ [d] cal/mole	$(\delta n_i)(\Delta F_{t(i)})$ kcal/mole
(B) Nonpolar					
Aliphatic					
Gly	21	24 (MV)	3 (MV)	4 (MV)	0 (MV)
Ala	41	34 (CC) 35 (MV) 32 (CE)	−7 (CC) −6 (MV) −9 (CE)	33 (CC) 9 (MV) 20 (CE)	−0.2 (CC) −0.1 (MV) −0.2 (CE)
Val	17				
Ileu	15	10 (CE) 18 (ML50)	−5 (CE) 3 (ML50)	−150 (CE) −196 (ML50)	0.8 (CE) −0.6 (ML50)
Leu	24				
Pro [e]	9	11 (MV)	2 (MV)	–	–
Met	8	5 (ML50) 3 (AV)	−3 (ML50) −5 (AV)	−300 (ML50) −182 (AV)	0.9 (ML50) 0.9 (AV)
1/2-Cys	2	4 (ML37) 4 (MV) 6 (CC)	2 (ML37) 2 (MV) 4 (CC)	−110 (ML37) −110 (MV) −110 (CC)	−0.2 (ML37) −0.2 (MV) −0.4 (CC)
Aromatic					
Tyr	13	11 (CE) 11 (PC)	−2 (CE) −2 (PC)	−390 (CE) −376 (PC)	0.8 (CE) 0.8 (PC)
Phe	8	10 (PL)	2 (PL)	−300 (PL)	−0.6 (PL)

a) Abbreviations signify algal source of phycocyanin. PC, *Plectonema calothricoides*; PL, *Phormidium luridum*; CC, *Cyanidium caldarium*; MV, *Microcoleus vaginatus*; CE, *Coccochloris elabens*; ML37, *Mastigocladus laminosus* (I-30-m); ML50, *Mastigocladus laminosus* (NZ-DB2-m); AV, *Anabaena variabilis*.

b) Amino acid residues are arbitrarily idealized composite values based on a molecular weight of 30,000 for phycocyanin monomer. They are used as reference values. Sources of data: ML37, ML50 and PC [5], CC [33], AV [6], PL [9], MV [8] and CE [7]. Experimental error is taken as ± 7%, therefore the calculations where $|\delta n_i| <$ the reference number of residue × 7% are neglected in the table.

c) Deviation in the number of amino acid residue i from the reference value.

d) Free energy of transfer from water to urea solution. The concentration of urea solution is assigned at $(C_u)_{1/2}$ except that for 1/2-Cys.

e) $\Delta F_{t(i)}$ of Lys and Pro are not available. Based on ref. [25], the estimated values of $(\delta n_i)(\Delta F_{t(i)})$ of Lys for CE and Pro for MV would be in the range of 0.2 kcal/mole which will not significantly effect the calculated $\Delta G^{H_2O}_{app(c)}$.

f) The magnitude of $\Delta F_{t(i)}$ is small based on the assumption of Ser being equivalent to Thr–Ala [26].

for the extra stability of psychrophilic phycocyanin at this stage is unknown. It was found that the purified psychrophilic phycocyanin contains a significant amount of polysaccharide [8]. It is not clear as to whether all the polysaccharide found is simply a contaminant or if a small amount is part of the protein structure (a glycoprotein). It is possible that this material could interact with protein or urea so as to affect the stability of protein. A variety of enzymes from extremely halophilic bacteria have been shown to require high concentrations of Na^+ or other cations for both activity and stability [16]. The mechanism of action of salts has been proposed to involve shielding of ionic groups on the enzyme by counterions [31]

Table 4
Aggregation states of phycocyanin in pH 6.0, I 0.1 phosphate buffer at 25°C

Source of phycocyanin	Percentage of aggregates [a]						Degree of polymerization [b]
	21S	19S	16S	11S	6S	3S	
Thermophile							
M. laminosus (I-30-m)		2		43	55		4.5
M. laminosus (NZ-DB2-m)		12		29	59		5.0
C. caldarium		46		19	35		7.7
Mesophile							
A. variabilis		37		43	20		7.6
P. calothricoides		5		70	25		5.6
P. luridum		7		32	62		4.6
Psychrophile							
M. vaginatus	11 [c]		13 [c]	26	19	30	5.3
Halophile							
C. elabens		7		27	66		4.4

a) 19S = dodecamer, 11S = hexamer, 6S = trimer, and 3S = monomer. Data taken from refs. [5, 7, 8, 33–35].
b) Degree of polymerization = 12 × percentage of 19S + 6 × percentage of 11S + 3 × percentage of 6S + 1 × percentage of 3S.
c) Arbitrary assignment of the sum of the amount of 21S and 16S aggregates as being equal to the amount of 19S aggregate.

and salting out of nonpolar side chains allowing them to form stable hydrophobic bonds within the interior of the protein structure. For sodium or potassium salts, the order of promoting enzyme activity and stability is Cl^-, $H_2PO_4^-$ > Br^- > NO_3^- > ClO_4^- > SCN^- [17]. In the present study, the purified halophilic phycocyanin is dissolved in sodium phosphate buffer. The high stability of $\Delta G_{app}^{H_2O}$ for halophile in the present study might result from the specific interactions of the protein with sodium and phosphate ions.

$\Delta G_{app(c)}^{H_2O}$ is the apparent free energy of protein unfolding at zero denaturant concentration corrected for differences in amino acid content. Table 2 shows that average values of $\Delta G_{app(c)}^{H_2O}$ are about the same for thermophile (4.6 kcal/mole), mesophile (4.3 kcal/mole), and psychrophile (4.7 kcal/mole), while that of halophile is much higher. These results suggest that the difference observed in $\Delta G_{app}^{H_2O}$ among these proteins are primarily due to the minor difference in amino acid compositions and also suggest the possibility that there is a specific interaction of halophile with the ions in the solution so as to give a much higher $\Delta G_{app(c)}^{H_2O}$.

The aggregation state of phycocyanin to a significant extent results from protein-protein and protein-solvent interactions [9, 10]. In analyzing the data shown in table 4, one will find no systematic correlation between the percentages of aggregates or the degree of polymerization and the sources of the proteins. It is possible that the minor difference in amino acid compositions would provide a difference in protein-protein and protein-solvent interactions. However, it is not clear how this amino acid difference is related to the aggregation states of phycocyanins, even though it has been shown that a single amino acid residue substitution can dramatically affect the aggregation properties and physiological function of hemoglobin [32].

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