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Biophysical Chemistry 60 (1996) 89–97

Biophysical Chemistry

Comparative thermodynamic elucidation of the structural stability of thermophilic proteins

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Received 25 September 1995; revised 4 January 1996; accepted 10 January 1996

Abstract

Differential scanning calorimetry, circular dichroism, and visible absorption spectrophotometry were employed to elucidate the structural stability of thermophilic phycocyanin derived from *Cyanidium caldarium*, a eucaryotic organism which contains a nucleus, grown in acidic conditions (pH 3.4) at 54°C. The obtained results were compared with those previously reported for thermophilic phycocyanin derived from *Synechococcus lividus*, a procaryote containing no organized nucleus, grown in alkaline conditions (pH 8.5) at 52°C. The temperature of thermal unfolding (t_d) was found to be comparable between *C. caldarium* (73°C) and *S. lividus* (74°C) phycocyanins. The apparent free energy of unfolding ($\Delta G_{[urea]=0}$) at zero denaturant (urea) concentration was also comparable: 9.1 and 8.7 kcal/mole for unfolding the chromophore part of the protein, and 5.0 and 4.3 kcal/mole for unfolding the apoprotein part of the protein, respectively. These values of t_d and $\Delta G_{[urea]=0}$ were significantly higher than those previously reported for mesophilic *Phormidium luridum* phycocyanin (grown at 25°C). These findings revealed that relatively higher values of t_d and $\Delta G_{[urea]=0}$ were characteristics of thermophilic proteins. In contrast, the enthalpies of completed unfolding (ΔH_d) and the half-completed unfolding $(\Delta H_d)_{1/2}$ for *C. caldarium* phycocyanin were much lower than those for *S. lividus* protein (89 versus 180 kcal/mole and 62 versus 115 kcal/mole, respectively). Factors contributing to a lower ΔH_d in *C. caldarium* protein and the role of charged groups in enhancing the stability of thermophilic proteins were discussed.

Keywords: Thermophilic proteins; Thermodynamic elucidation; Structural stability

1. Introduction

The stability of thermophilic proteins has been a subject of interest for many years [1–6]. Studying their properties is a natural approach to investigate the structure and stability of proteins in general [1,2,7–12]. Proteins isolated from thermophilic microorganisms exhibit greater resistance to thermal and chemical denaturation than proteins from mesophilic ones [1,2]. Thermostable enzymes retain a high degree of specificity and possess a practical advantage owing to their operational stability [13]. Comparative study of the structural stability of thermophilic proteins with that of less stable counterparts (mesophilic proteins) not only can provide valuable insights into factors responsible for the stabilization of biomolecules, but also can supply useful knowledge on how to purposefully make proteins more stable [14–16]. Moreover, investigation of

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PII S0301-4622(96)00013-0

thermophilic proteins furnishes an opportunity to examine how nature adapts protein structure to various extreme environmental conditions [3–6].

A useful probe for studying the differences in the structure and conformation of proteins in response to variations in environmental conditions is phycocyanin, which is the major protein in cyanophytes. Phycocyanin is an accessory pigment in photosynthesis. It does not exhibit enzymatic activity in the conventional sense, and exists in a diversity of ecological niches under vastly different environmental stresses, such as temperature and salt conditions [2]. The temperature adaptation observed in the structure of phycocyanin makes the protein a useful model protein in the elucidation of the factors underlying the structural stability of thermophilic proteins.

We previously found that thermophilic phycocyanins resist high temperature and denaturants, and retain much of their native structure at temperatures where corresponding mesophilic proteins are denatured. This characteristic of higher resistance to denaturation is analogous to other thermophilic proteins such as 3-phosphoglycerate kinase [8–10], malate dehydrogenase [6,13], and malate synthase [12]. On the basis of free energy of unfolding, we found that the ability of various phycocyanins to resist the denaturant urea is in the order of thermophile > mesophile > psychrophile [2]. Among phycocyanins from a diverse group [17,18], only minor differences in amino-acid compositions, amino-acid sequences, and immunochemistry were found. The free energy of unfolding of phycocyanins was found to be correlated with minor amino acid differences between thermophilic and mesophilic proteins [2]. These observations supported the assertion that minor differences in amino-acid composition can cause significant alteration in the structure and stability of proteins [2,4,6,14,15], as were seen in an increased thermostability due to three amino-acid replacements in bacillar alcohol dehydrogenase [19], and by an increasing hydrophobic index through enhancing the ratio of Arg or (Arg + Lys) to total amino acids as seen in thermophilic protein from genus Bacillus [4].

In a recent study of thermophilic phycocyanin derived from thermophilic cyanobacteria, *S. lividus* II [1], we found that the enthalpy of thermal unfolding of *S. lividus* phycocyanin is much higher than that of a mesophilic protein derived from *P. luridum*. In contrast, the free energy of denaturant unfolding in *S. lividus* protein is only modestly higher as compared to *P. luridum* protein. This difference was attributed to a higher entropy of unfolding in thermophilic phycocyanin, which could be derived from a more rigid overall structure that possesses higher internal hydrophobicity and stronger internal packing. Improved packing densities has been shown to be crucial to the enhanced thermostability in thermophilic proteins [20].

Cyanobacterium *S. lividus* used in the above study is an archaebacterium which contains no organized nucleus; it grows in alkaline conditions (pH 8.5). Another microorganism available is *Cyanidium caldarium*, a more complex organism containing a nucleus, which grows in acidic conditions (pH 3.5). Despite these differences, these two microorganisms are thermophilic and grow in a comparable temperature range (52–58°C). In extension of our previous studies of thermophilic phycocyanins [1,2], and to obtain a deeper understanding of the nature of the thermostability of thermophilic proteins, we have employed differential scanning calorimetry, circular dichroism, and visible absorption spectrophotometry to explore the structural stability of thermophilic phycocyanin derived from *C. caldarium*. The results were compared with those previously reported for thermophilic phycocyanin derived from *S. lividus* [1]. This work was part of a continuing investigation to determine whether the relatively higher temperature of thermal unfolding and free energy of unfolding, encountered when comparing phycocyanins from *S. lividus* and *P. luridum*, would be a characteristic of the additional structural stability of thermophilic phycocyanins. In this study, it was demonstrated that the temperature and free energy effects exist in phycocyanin from *C. caldarium*; however, there is a profound difference in the enthalpy of thermal unfolding for this thermophilic protein.

2. Experiments

2.1. Microorganism and proteins

Thermophilic *C. caldarium* was grown at 54°C in Allen's medium, pH 3.5. The harvested cells were

kindly provided by Dr. Robert MacColl of the Wadsworth Center. Cells were lysed with lysozyme. Then the major biliprotein, phycocyanin, was extracted in sodium phosphate buffer (pH 6.0, 0.1 ionic strength), the same buffer solution used to isolate thermophilic phycocyanin from *S. lividus*. These two thermophilic phycocyanins could then be compared under the same conditions.

The extracted protein was purified according to known procedures [2,17,18]: protein solution was repeatedly precipitated with 50% ammonium sulfate and redissolved in pH 6.0 buffer medium; the protein was then dialyzed in 35% ammonium sulfate solution and fractionated several times until the ratio of A_{620} to A_{280} was close to 4. The concentration of protein was determined on the basis of a specific extinction coefficient of 6.0 at 620 nm for 1 mg/ml of protein [2,17,18].

2.2. Urea solution

Ultra pure-grade urea was obtained from Schwarz/Mann Co. Urea solutions from 1 to 10 M in 1 M or 0.5 M increments were prepared by dissolving appropriate amounts of urea in pH 6, sodium phosphate ($I = 0.1$) buffer. The addition of urea to the buffer solution resulted in only a small change in the pH of the solution (for instance, the pH increased slightly from 6.0 to 6.3 in 7 M urea); however, this minor change in the pH of the protein solution as urea was added was not readjusted since it was necessary to maintain the same reference state.

2.3. Differential scanning calorimetry (DSC)

Analogous to our previous thermodynamic studies of proteins [1,22–26], thermal unfolding of phycocyanin was carried out with a Hart Scientific DSC according to the previous procedures [1]. Protein sample (0.20 ml; 3.5 to 5.1 mg/ml) in the absence and presence of an appropriate concentration of urea ([urea]) was scanned from 20 to 90°C at a heating rate of 40°C or 60°C/h. Control measurements were made with buffer solution in the absence and presence of comparable urea concentrations. For purposes of data comparison between various runs, the raw data files from the DSC were transferred to a Macintosh computer. Baseline manipulations were carried out using a statistical program called Statview, to produce a common baseline starting point at 30°C in the denaturation thermograms. The data files were then transferred back to the calorimeter which had software for baseline subtraction, peak area integration, unit conversion, etc. Adjusting the starting point made it easier to compare results and do baseline subtraction. Comparisons were made between modified and unmodified data files to assure that no artifacts were introduced by these manipulations.

2.4. Visible absorption spectrophotometry

A Perkin-Elmer lambda 4B UV/Visible spectrophotometer was used to measure absorption spectra from 450 to 710 nm as a function of [urea] at room temperature. Phycocyanins have a maximum absorption at around 620 nm. About 30–40 μL of phycocyanin solution was added to 2–5 ml buffer solution to give a final solution with an optical density about 0.8–0.9 for absorbance measurements at this wavelength. With the addition of urea, the absorbance decreased as time increased and then leveled off [2]. The mixed protein–urea solutions were equilibrated for 30 min and were monitored over this time period to ascertain that no additional changes were occurring. This ensured that the denaturation process was completed before measurements were taken.

2.5. Circular dichroism (CD)

A Jasco J-720 spectropolarimeter was used to obtain CD spectra of phycocyanin solutions in the presence of 0 to 10 M urea, using CD cells with a 0.1-cm light path. The CD spectra of phycocyanin showed a strong positive band at around 630 nm and a strong negative band at 330 nm [26,27]. It is generally accepted that changes in protein conformation are reflected in the spectral region from 198 to 230 nm [2,28,29]; in the investigation of the secondary structure of phycocyanin, CD measurements in the presence of 0 to 10 M urea were made in that region to study the free energy of protein unfolding. These CD data ranging in 1-nm intervals were fitted by an internal software program.

3. Results

3.1. Thermal unfolding of thermophilic proteins by differential scanning calorimetry (DSC)

Phycocyanins are complex assemblies of protein subunits, the subunits being composed of apoproteins (polypeptides) and chromophores (tetrapyrroles or bilins). As these proteins undergo thermal unfolding, the structure of the apoproteins unfolds from helical to coil conformation, and that of the chromophores changes from linear to cyclic conformation. Typical measurements of thermal unfolding of *C. caldarium* phycocyanin, as determined by DSC, are presented in Fig. 1. The figure shows that, in the absence of urea, the unfolding thermogram of the protein exhibits two domains (double peaks) having maxima at 76°C and 69°C, respectively (Fig. 1a). In the presence of 1 M urea (Fig. 1b), the two domains become less separated and look more or less like one broad peak ranging from 67 to 74°C. As [urea] increases to 3 M or higher (Fig. 1c–e), the thermograms exhibit a single peak, and the peaks become smaller and shift to a lower temperature range.

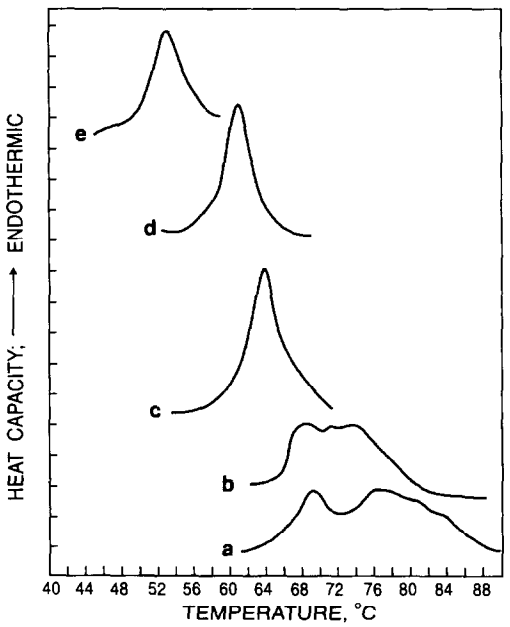

Fig. 1. Typical differential scanning calorimetric measurements of thermal unfolding of thermophilic phycocyanin from *C. caldarium* in the absence and presence of urea: (a) no urea, (b) 1 M urea, (c) 3 M urea, (d) 4 M urea, and (e) 5 M urea. Each measurement involved 0.20 ml of 5 mg/ml of protein.

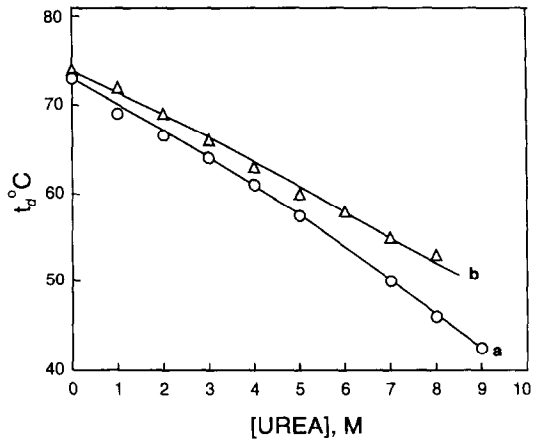

Fig. 2. Temperature of thermal unfolding of phycocyanin as a function of urea concentration: (a) thermophilic phycocyanin from *C. caldarium* and (b) thermophilic phycocyanin from *S. lividus*. Data of *lividus* phycocyanin are derived from Ref. [1].

Values of the thermal unfolding temperature (t_d) of *C. caldarium* protein as a function of [urea] are plotted in Fig. 2a, where t_d is determined by the peak temperature or by the temperature at which the unfolding is half-way completed. The figure shows that the magnitude of t_d decreases as [urea] increases. For comparison, values of t_d for *S. lividus* phycocyanin are plotted in Fig. 2b. The figures reveal that at a corresponding [urea] ranging from 0 to 8 M, the magnitude of t_d for *C. caldarium* protein is lower that for *S. lividus* protein. The difference ranges from 1 to 7 degrees, and becomes larger as [urea] increases. For *C. caldarium* protein, the value of t_d decreases from 73°C to 46°C as [urea] increases from 0 to 8 M, while it decreases from 74°C to 53°C for *S. lividus* protein. The results indicate that the degree of urea effect on t_d is −3.4°C/mole of urea for the former protein, as compared with −2.5°C/mole of urea for the latter protein.

Table 1 reveals that, in the absence of urea, the enthalpy of thermal unfolding (ΔH_d) for *C. caldarium* protein is 89 kcal/mole, as compared with 180 kcal/mole for *S. lividus* protein [1]. This finding is significantly different from that for t_d, which has comparable values for the two proteins. The dependence of ΔH_d on [urea] for *C. caldarium* protein is presented in Fig. 3a, which depicts three regions

Table 1
Comparison of thermal unfolding of thermophilic phycocyanins

Microroganism	t_d, °C	ΔH_d, kcal/mole	$\Delta H_{d(1/2)}$, kcal/mole	$(C_u)_{1/2}$, mole/l [a]
In the absence of urea				
C. caldarium	73	89		
S. lividus	74	180		
P. luridum	63	98		
In the presence of urea				
C. caldarium			62	5.0
S. lividus			115	3.4
P. luridum			47	3.6

[a] Values were determined by differential scanning microcalorimetry. Experimental error is ±1°C for t_d, no greater than 10% for (ΔH_d) and (ΔH_d)$_{1/2}$, and ±0.2 mole/l for $(C_u)_{1/2}$.

representing the protein before unfolding, while undergoing unfolding, and after unfolding, respectively. The magnitude of ΔH_d decreases in the presence of urea. The denaturation curve drops sharply from 3 to 7 M urea, and slowly levels off after 7 M urea. From the denaturation curve and the extrapolated straight lines before and after the denaturation, the concentration of urea at which the thermal unfolding of protein is half-completed, $(C_u)_{1/2}$, is found to be 5.0 M, and the corresponding enthalpy change, $\Delta H_{d(1/2)}$, is 62 kcal/mole, as compared to 3.4 M for $(C_u)_{1/2}$ and 115 kcal/mole for $\Delta H_{d(1/2)}$ in *S. lividus* protein (Table 1). For comparison, the dependence of ΔH_d on [urea] for *S. lividus* phycocyanin is also plotted in Fig. 3b. Data of t_d, ΔH_d, $(C_u)_{1/2}$ and $\Delta H_{d(1/2)}$ for *C. caldarium*, *S. lividus* and *P. luridum* proteins are compared in Table 1.

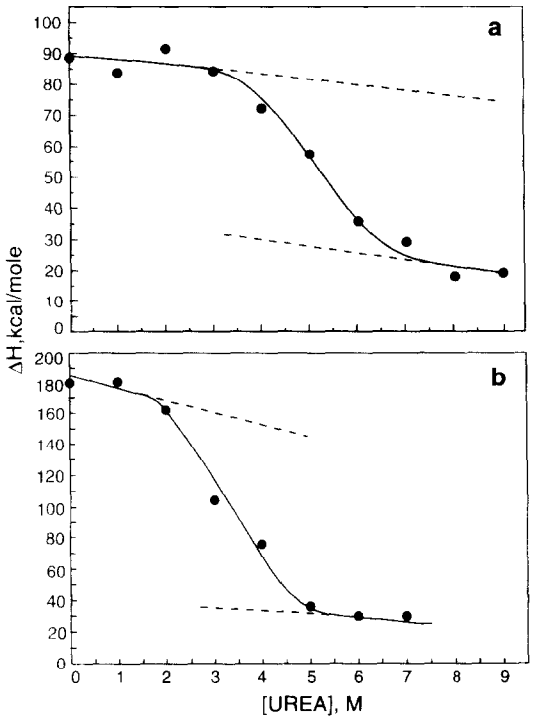

Fig. 3. The enthalpy of thermal unfolding (ΔH_d) of phycocyanins as a function of urea concentration: (a) thermophilic phycocyanin from *C. caldarium*, and (b) thermophilic phycocyanin from *S. lividus*. See Fig. 2 for other conditions.

3.2. Unfolding of thermophilic phycocyanin as measured by circular dichroism (CD)

In the denaturation of phycocyanin monitored by CD, the structure of apoproteins in phycocyanin unfolds from helical to coil conformation. The CD spectrum of phycocyanin has a strong positive band at 630 nm and a strong negative band at 330 nm [26,27]. In the range from 240 to 199 nm, the protein exhibits two shoulders at 220 and 208 nm [23,24]. The denaturation profile of *C. caldarium* protein in urea solution is shown in Fig. 4a, which plots the dependence of CD at 222 nm on [urea]. In analogy to Fig. 3a, Fig. 4a consists of three regions representing the protein before unfolding, while undergoing unfolding, and after unfolding, respectively. From the figure, the [urea],$(C_u)_{1/2}$, at which the protein denaturation is half-completed is found to be 6.0 M, as compared with a value of 6.5 M for *S. lividus* protein (Table 2). For comparison, a similar plot for *S. lividus* protein is presented in Fig. 4b.

A two-state mechanism (Eq. (1)), originally proposed to explain the observed urea or guanidine hydrochloride denaturation profiles for ribonuclease, lysozyme, α-chymotrypsin and lactoglobulin [28–30], was appropriately applied to phycocyanins in our previous studies [1,2]. This assumption is also adopted in the present study to examine the free energy change in the unfolding of *C. caldarium* phycocyanin determined from the CD data. In analogy to previously reported *S. lividus* protein, the equilibrium constant (K) was determined from the data shown in Fig. 4a according to Eq. (2):

$$\text{Native state} \rightleftharpoons \text{Denatured state} \quad (1)$$

$$K = ([\text{CD}]_N - [\text{CD}])/([\text{CD}] - [\text{CD}]_D) \quad (2)$$

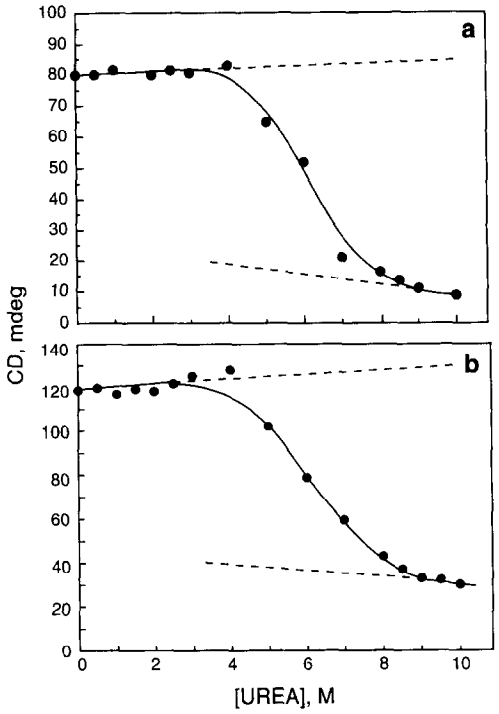

Fig. 4. Circular dichroism of phycocyanins at 222 nm as a function of urea concentration: (a) thermophilic phycocyanin from *C. caldarium*, and (b) thermophilic phycocyanin from *S. lividus*. Data of *lividus* phycocyanin are derived from Ref. [1]. Protein concentration = 0.12 mg/ml.

where [CD] denotes the observed circular dichroism at 222 nm for protein in urea solution, and $[CD]_N$ and $[CD]_D$ are those which the native and the completely unfolded proteins would have under the same conditions. The magnitudes of $[CD]_N$ and $[CD]_D$ in the region where the protein is undergoing unfolding can be obtained from the denaturation curve and the extrapolated straight lines as shown in Fig. 4a.

From the determined values of K, the apparent free energy of denaturation, ΔG^{α}, can be calculated according to

$$\Delta G^{\alpha} = -RT \ln K \quad (3)$$

To obtain the apparent free energy of unfolding at zero denaturant (urea) concentration, $\Delta G_{[urea]} = 0$, data of ΔG^{α} were fitted to the equation

$$\Delta G^{\alpha} = \Delta G_{[urea]=0} - m[urea] \quad (4)$$

where m is a constant. A value of 5.0 kcal/mole for $\Delta G_{[urea]=0}$ is found for *C. caldarium* protein (Table 2). This value is slightly higher than that previously obtained, 4.3 kcal/mole, for *S. lividus* protein. The value of m (not listed in the table) is found to be 0.9 for *C. caldarium* phycocyanin, which is comparable to 0.8 for *S. lividus* protein and 1.1 for ribonuclease and lysozyme [29], where a two-state mechanism is also adopted.

CD data in the absence of urea ranging from 232 to 198 nm were used to determine the secondary structure of *C. caldarium* phycocyanin. The results are also listed in Table 2. The table indicates that the secondary structure of *C. caldarium* protein has 50% α-helix, 27% β-sheet and β-turn, and 24% random coil. This secondary structure is comparable to that of *S. lividus* protein [1].

3.3. Unfolding of thermophilic phycocyanin as measured by visible absorption spectrum

Denaturant unfolding of *C. caldarium* protein was also examined using visible absorption data at

Table 2
Circular dichroism and visible absorption spectrophotometric studies of urea unfolding of phycocyanins

Microorganism	$(C_u)_{1/2}$, mole/l	$\Delta G_{[urea]=0}$, kcal/mol	Secondary structure			
			α-helix %	β-sheet %	β-turn %	random %
C. caldarium	6.0 [a] (5.7) [b]	5.0 [a] (9.1) [b]	50	5	22	24
S. lividus	6.5 (6.5)	4.3 (8.7)	52	7	20	21
P. luridum	4.5 (4.7)	4.0 (5.3)	55	3	20	22

[a] Values (not in parentheses) were determined by CD at 222 nm.
[b] Values (in parentheses) were determined by visible absorption at 620 nm. Experimental error was estimated as ± 0.2 mole/l for $(C_u)_{1/2}$, ± 0.3 kcal/mole for ΔG, and about $\pm 2\%$ for secondary structure determination.

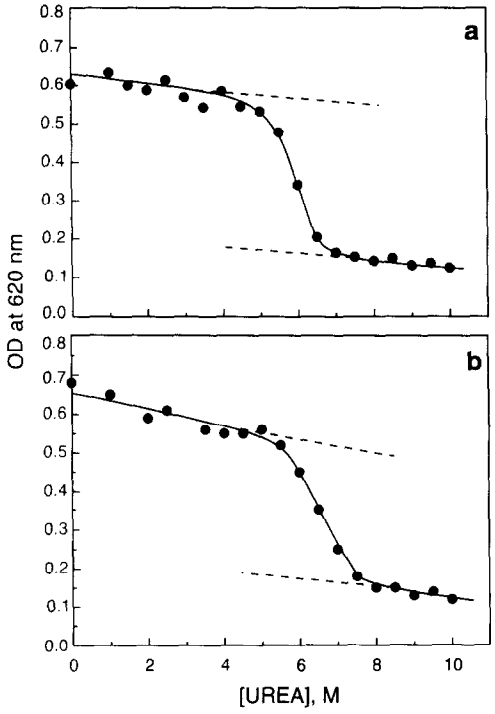

Fig. 5. The visible absorption maximum of phycocyanins at 620 nm as a function of urea concentration: (a) thermophilic phycocyanin from *C. caldarium*, and (b) thermophilic phycocyanin from *S. lividus*. Data of *S. lividus* phycocyanin are derived from Ref. [1]. Protein concentration = 0.16 mg/ml.

620 nm (the maximum absorption wavelength) in a manner similar to CD experiments. Changes in the visible absorption spectrum reflects change in the chromophores of phycocyanin, from linear to cyclic conformation. A plot of absorbance versus [urea] is presented in Fig. 5. In the presence of 1–10 M urea, the absorbance decreases as [urea] increases. From the plot, the value of $(C_u)_{1/2}$ was determined. Table 2 shows that the value of $(C_u)_{1/2}$ is 5.7 M for *C. caldarium* protein, comparable to the value of 6.5 M previously reported for *S. lividus* protein. In analogy to the treatment of CD data (see Eq. (4)), values of m and $\Delta G_{[urea]=0}$ were also determined from the visible absorption spectrum. Table 2 shows that $\Delta G_{[urea]=0}$ has a value of 9.1 kcal/mole for *C. caldarium* protein, as compared with previously reported 8.7 kcal/mole for *S. lividus* protein. The value of m was found to be 1.5 (not shown in the table), which is higher than 0.9 as determined from CD data.

4. Discussion

Results of thermal unfolding of proteins as presented in Table 1 show that, in the absence of urea, the thermal unfolding temperature (t_d) is comparable between *C. caldarium* protein (73°C) and *S. lividus* protein (74°C). Both proteins exhibit a value of t_d about 10°C higher than mesophilic *P. luridum* phycocyanin. Table 2 also shows that, although the apparent free energy change at zero denaturant concentration, $\Delta G_{[urea]=0}$, for helical-coil apoprotein conformational change is slightly higher in *C. caldarium* protein (5.0 kcal/mole) than *S. lividus* protein (4.3 kcal/mole), the magnitude of $\Delta G_{[urea]=0}$ for linear–cyclic chromophore conformational change is comparable (9.1 and 8.7 kcal/mole, respectively). Consequently, the apparent free energy of unfolding of the whole protein at zero denaturant is comparable between these two thermophilic proteins and is significantly higher than that of mesophilic *P. luridum* protein. These findings suggest that relatively higher values of thermal unfolding temperature and free energy of unfolding are characteristic of thermophilic proteins.

In addition, the value of $(C_u)_{1/2}$, at which the urea unfolding of the apoprotein part of phycocyanin from helical to coil conformation is half-completed, exhibits no significant difference between *C. caldarium* and *S. lividus* proteins (6.0 M versus 6.5 M) (Table 2). A similar observation can be found in the urea unfolding of the chromophore part of phycocyanin from a linear to a cyclic conformation, which shows a comparable $(C_u)_{1/2}$ between the two proteins (5.7 M versus 6.5 M urea, respectively) (Table 2). These values of $(C_u)_{1/2}$ are higher than the corresponding data for *P. luridum* protein, confirming that thermophilic proteins in general are more resistant to thermal and urea denaturation than mesophilic proteins.

In contrast, the value of ΔH_d (the denaturation enthalpy change) for *C. caldarium* protein (89 kcal/mole) is much lower than that of *S. lividus* protein (180 kcal/mole). Similarly, the corresponding half-completed denaturation enthalpy change $(\Delta H_d)_{1/2}$, is also lower for *C. caldarium* protein (62 kcal/mole) than *S. lividus* protein (115 kcal/mole). These enthalpy values of *C. caldarium* protein at 73°C are not significantly different from those of *P.*

Table 3
Comparison of the percentage of polar residues to the total amino acids in thermophilic and mesophilic phycocyanins

Source of phycocyanin [a]	Acidic residue				Basic residue					
	Asp		Glu		Lys		His		Arg	
	number	%	number	%	number	%	number	%	number	%
Thermophilic										
C. caldarium [b]	28	10.2	25	9.1	11	4.0	2	0.7	18	6.5
S. lividus (SyI) [b]	27	9.5	30	10.6	10	3.5	3	1.1	20	7.0
Mesophilic										
P. luridum [c]	29	10.0	19	6.5	13	4.5	2	0.7	17	5.8
P. calothricoides [b]	30	11.2	19	7.1	11	4.1	2	0.7	15	5.6

[a] Total number of amino acid residues = 275, 284, 291 and 269 for *C. caldarium*, *S. lividus*, *P. luridum* and *P. calothricoides*, respectively.
[b] Data are taken from Ref. [21]
[c] Data are taken from Ref. [31]

luridum protein at 63°C. In addition to distinct values of ΔH_d in these two thermophilic proteins, the unfolding thermogram of *C. caldarium* phycocyanin exhibits double peaks (or two domains) as compared to one peak (single domain) in *S. lividus* protein [1].

The finding of a comparable free energy of unfolding between phycocyanins derived from *C. caldarium* and *S. lividus* versus a much lower enthalpy of unfolding for *C. caldarium* protein reveals a significant difference in the entropy of protein native state between the two phycocyanins. Since $\Delta H = \Delta G + T\Delta S$, $\Delta H_{cc} < \Delta H_{sl}$, $\Delta G_{cc} \approx \Delta G_{sl}$, and $T_{d,cc} \approx T_{d,sl}$, where cc and sl denote *C. caldarium* and *S. lividus* phycocyanins, respectively, therefore $\Delta S_{cc} < \Delta S_{sl}$. Furthermore, $\Delta S_{cc} = S_{u,cc} - S_{f,cc}$ and $\Delta S_{sl} = S_{u,sl} - S_{f,sl}$, where u and f denotes unfolded (denatured) and folded (native) states of phycocyanin, respectively. In view of a similarity in amino acid composition between phycocyanins [21], it was assumed that $S_{u,cc} \approx S_{u,sl}$. Consequently, $-S_{f,cc} < -S_{f,sl}$ or $S_{f,cc} > S_{s,sl}$, suggesting that in the native state *C. caldarium* phycocyanin has a higher entropy than that of *S. lividus* protein. A higher entropy in the native state of *C. caldarium* protein might be associated with the observation of two domains in its unfolding thermogram, as compared to a single domain in that of *S. lividus* protein. In contrast, the helical content was not expected to contribute to the entropy difference between these two thermophilic phycocyanins, since their secondary structure is similar (Table 2).

In spite of a difference in the entropy of the native state of *C. caldarium* and *S. lividus* phycocyanins, the data of t_d, $\Delta H_{d(1/2)}$, $(C_u)_{1/2}$, and $\Delta G_{[urea]=0}$ as shown in Tables 1 and 2 confirm that both thermophilic phycocyanins are more resistant to thermal stress than mesophilic *P. luridum* protein, and exhibit an increase in protein stability as an adaptation to the environmental stress of high temperature.

Comparison of the percentages of polar residues to the total amino acids in thermophilic and mesophilic phycocyanins are presented in Table 3. The table reveals that the percentages of Glu and Arg residues for thermophilic caldarium and lividus phycocyanins are significantly higher than those of mesophilic luridum and calothricoides phycocyanins (9.1 and 10.6% versus 6.5 and 7.1% for Glu and 6.5 and 7.0% versus 5.8 and 5.6% for Arg, respectively), although no significant difference is found for Asp, Lys and His residues between the two types of phycocyanin. A higher ratio of basic polar residues (Arg and Lys) was also found for thermophilic protein from genus (Bacillus) [4]. Our results suggest a possible enhanced stability of thermophilic phycocyanins due to a higher ratio of polar residues which increase charge interactions in proteins. In addition, phosphate ions in the buffer medium may also be involved in charge interactions [32], leading to fur-

ther stabilization of the intrinsic structure of thermophilic proteins.

Acknowledgements

The authors gratefully acknowledge the use of Wadsworth Center's Biochemistry Core facilities.

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