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Biophysical Chemistry 50 (1994) 313–321

Biophysical Chemistry

Thermodynamics elucidation of the structural stability of a thermophilic protein

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(Received 24 August 1993; accepted in revised form 6 December 1993)

Abstract

The structural stability of the protein, phycocyanin isolated from two strains of cyanophyta, *Synechococcus lividus* (thermophile) and *Phormidium luridum* (mesophile), are investigated by comparative thermal and denaturant unfolding, using differential scanning calorimetry, visible absorption spectrophotometry, and circular dichroism. The thermophilic protein exhibits a much higher temperature and enthalpy of unfolding from the native to the denatured state. The concentration of urea at half-completion of thermal unfolding is essentially the same between the thermophilic and mesophilic proteins; in contrast, the corresponding temperature and the enthalpy of thermal unfolding are much higher for the thermophilic protein. In addition, the concentration of urea at which the non-thermal (denaturant) unfolding of protein is half-completed, as detected by either circular dichroism or absorption spectroscopy, is significantly higher in the thermophilic protein, while the apparent free energy of unfolding only shows a moderate difference between the two proteins. The distinct differences in the enthalpy of thermal unfolding and the free energy of denaturant unfolding are interpreted in terms of a significant entropy change associated with the unfolding of these proteins. This entropy contribution is much higher in the thermophilic protein, and may be derived from its more rigid overall structure that possesses higher internal hydrophobicity and stronger internal packing.

Key words: Phycocyanin; Thermal unfolding; Differential scanning calorimetry; Visible absorption spectrophotometry; Circular dichroism

1. Introduction

Comparison of the properties of thermophilic proteins and those of less stable counterparts from mesophilic microorganisms can provide (a) a natural approach to the investigation of protein stability [1–7], (b) an opportunity to study the mechanism of how nature adjusts protein structure to extreme environmental conditions [8–11], and (c) insight into making proteins more stable by chemical modification or site-directed mutagenesis for biotechnological applications [12–14]. The basis for resistance to denaturation in thermophilic proteins has been a subject of interest for many years [8–11].

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SSDI 0301-4622(93)E0102-B

Most of the proteins isolated from thermophilic microorganisms, such as 3-phosphoglycerate kinase [3–5], malate dehydrogenase [6], and malate synthase [7], resist high temperature and denaturants, and retain much of their native structure at temperatures where corresponding mesophilic proteins are denatured. Our previous investigations of the stability of phycocyanins isolated from thermophilic, mesophilic, and psychrophilic microorganisms showed that the ability of these proteins to resist the denaturant urea is in the order of thermophile > mesophile > psychrophile [1].

Protein thermostability can be enhanced by amino-acid substitutions [12,13]. Minor difference in amino-acid compositions can cause significant alteration in protein structure [1,9,11]. For instance, thermophilic versus mesophilic proteins from the same genus (Bacillus) exhibit correlations between thermostability and increased hydrophobic index and an increased ratio of Arg or (Arg + Lys) to total amino acids [9]. Amino-acid analyses, amino-acid sequences, and immunochemical studies of phycocyanins from a diverse group of cyanophyta demonstrated that these proteins exhibit a striking degree of similarity [15,16]. Moreover, the free energy of unfolding of phycocyanins is correlated with amino acid differences among proteins [1].

Phycocyanin is an energy-transfer pigment in Photosystem II [17], and is a modifier of electron flow across artificial chloroplast membranes [18]. Enzymatic activity in the conventional sense is not present in this protein. Hydrophobic interactions are believed to play a major role in its stability and aggregation state [19,20]. Since cyanophyta exist in a diversity of ecological niches and consequently thrive under vastly different environmental stress, their major protein, phycocyanin, can be used as a probe in the studies of the structure and conformation of proteins in response to variations in environmental conditions, such as temperature and salt conditions [1].

Our previous study on thermophilic proteins was limited to examining the free energy of protein unfolding. To understand better the structural stability of thermophilic proteins requires knowing the enthalpy or the entropy of unfolding in addition to the free energy. To extend our study of the thermodynamics of proteins [18–23] and their response to variations in environmental conditions [1], we have carried out both thermal and denaturant (urea) unfolding of thermophilic and mesophilic phycocyanins, using differential scanning calorimetry, circular dichroism, and visible absorption spectrophotometry. The results provide new insights into the thermostability of thermophilic proteins.

2. Experiments

2.1. Proteins

Thermophilic *Synechococcus lividus* (*S. lividus*) and mesophilic *Phormidium luridum* were grown at 52°C and 25°C, respectively, in specific media. After harvesting, the cells were lysed with lysozyme. Their major biliprotein, phycocyanin, was extracted in pH 6.0, 0.1 ionic strength (I), sodium phosphate buffer, and purified according to known procedures [1,15,16]. The protein solutions were repeatedly precipitated with 50% ammonium sulfate and redissolved in the 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 phycocyanin was determined on the basis of a specific extinction coefficient of 6.0 at 620 nm for 1 mg/ml of protein [1,15,16].

2.2. Urea solution

Ultra-pure grade urea was obtained from Schwarz/Mann Co. Urea solutions were prepared by dissolving appropriate amounts of urea in pH 6, $I = 0.1$, sodium phosphate 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). Since it was necessary to maintain the same reference state, such a small change in the pH of the protein solution, as urea was added, was not readjusted.

2.3. Differential scanning calorimetry (DSC)

Thermal unfolding of proteins was determined using a Hart Scientific DSC. The calorimeter had three sample cells and one reference cell. The original cells were modified by the insertion of a glass liner to eliminate the possibility of a reaction between the cells and sample solutions. 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/h. In the control measurements, the buffer solution in the absence and presence of urea was used. The DSC had software for baseline subtraction, peak area integration, unit conversion, etc. For purposes of data comparison between various runs, the raw data files from DSC were transferred to a Macintosh computer. Baseline manipulations were carried out using a statistical program called Statview, so that a common starting point at 30°C was shared among denauration thermograms. The data files were then transferred back to the calorimeter. 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 baseline manipulations.

2.4. Visible absorption spectrophotometry

The absorption spectra were measured in the ranges from 450 to 710 nm as a function of [urea], using a Perkin-Elmer lambda 4B UV/Visible spectrophotometer at room temperature. Phycocyanins have a maximum absorption at approximately 620 nm. For absorbance measurements at this wavelength, 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. With the addition of urea, the absorbance decreased as time increased and then leveled off [1]. To make sure that the denaturation process was completed before measurements were taken, 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.

2.5. Circular dichroism (CD)

CD spectra of phycocyanin solutions in the presence of 0 to 10 M urea were measured with a Jasco J-720 spectropolarimeter. The CD cells had 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 [24,25]. In the investigation of the secondary structure of phycocyanin, CD measurements were made in the spectral region from 198 to 230 nm. It is generally accepted that changes in protein conformation are reflected here [1,26,27]. These CD data ranging in 1 nm intervals were fitted by a software program. To study the free energy of protein unfolding by urea, CD measurements were also performed at 222 nm for protein solutions in the presence of 0 to 10 M urea.

3. Results

3.1. Thermal unfolding of thermophilic proteins

Biliproteins such as phycocyanins are complex assemblies of protein subunits, the subunits being composed of apoproteins (polypeptides) and chromophores (tetrapyrroles or bilins). As proteins undergo thermal denaturation, the structure of apoproteins unfolds from helical to coil conformation, and that of chromophores changes from linear to cyclic conformation. Thermal unfolding of phycocyanins isolated from *S. lividus* (thermophile) and *P. luridum* (mesophile), as determined by differential scanning calorimetry, exhibits a single transition having maxima at 74°C and 63°C, respectively. Typical unfolding thermograms in the absence and presence of urea are shown in Figs. 1 and 2, respectively. In the absence of urea, the temperature at which the thermal denaturation begins (t_1) and ends (t_2) and the peak temperature of each thermogram (t_d) are compared in Table 1 which reveals higher values of t_1, t_2 and t_d for thermophilic protein.

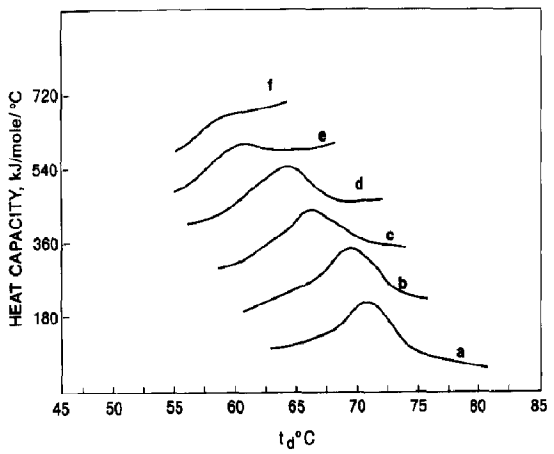

Fig. 1. Typical differential scanning calorimetric measurements of thermal unfolding of thermophilic phycocyanin from *S. lividus* in the absence and presence of urea: (a) no urea, (b) 1 M urea, (c) 2 M urea, (d) 3 M urea, (e) 4 M urea, and (f) 5 M urea. Each measurement involved 0.20 ml of 5 mg/ml of protein.

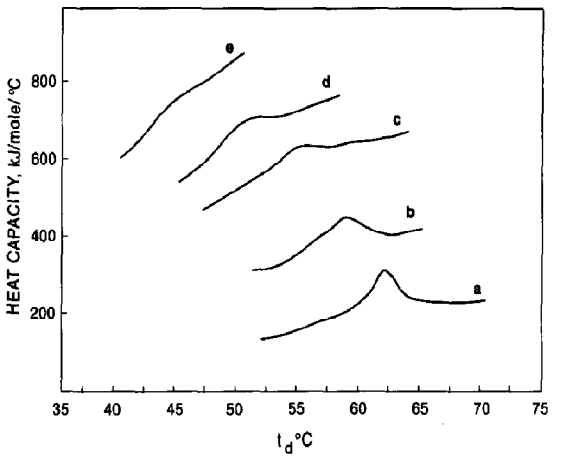

Fig. 2. Typical differential scanning calorimetric measurements of thermal unfolding of mesophilic phycocyanin from *P. luridum* in the absence and presence of urea: (a) no urea, (b) 1 M urea, (c) 2 M urea, (d) 3 M urea, and (e) 4 M urea. Each measurement involved 0.20 ml of 5 mg/ml of protein.

As [urea] increases, the thermograms become smaller and shift to a lower temperature range. At each [urea], the value of t_d for the thermophilic protein is significantly higher than that for the mesophilic one. The value of t_d decreases from 74°C to 53°C as [urea] increases from 0 to 8 M for the thermophilic protein, while it decreases from 63°C to 46°C as [urea] increases from 0 to 4 M for the mesophilic protein. These data indicate that the degree of urea effect on t_d is −2.5°C/mol of urea for the thermophilic protein, as compared with −4.2°C/mol of urea for the mesophilic protein. As expected, these results demonstrate more resistance to urea denaturation for the thermophilic than the mesophilic protein.

Table 1 also reveals that the enthalpy of thermal unfolding (ΔH_d) in the absence of urea is

Table 1
Differential scanning calorimetric study of thermal unfolding of thermophilic and mesophilic phycocyanins

Protein	Strain of cyanophyta	t_1 (°C)	t_2 (°C)	t_d (°C)	ΔH_d (kJ/mol)
in the absence of urea [a]					
thermophilic phycocyanin	*S. lividus*	65	88	74	753
mesophilic phycocyanin	*P. luridum*	56	72	63	414
Protein	**Strain of cyanophyta**	$(C_u)_{1/2}$ (mol/l)	$t_{d(1/2)}$ (°C)	$\Delta H_{d(1/2)}$ (kJ/mol)	
in the presence of urea [b]					
thermophilic phycocyanin	*S. lividus*	3.4	65	481	
mesophilic phycocyanin	*P. luridum*	3.6	48	197	

[a] Experimental error is ±1°C for t and no greater than 10% for ΔH_d.
[b] Experimental error is ±0.2 mol/l for $(C_u)_{1/2}$, ±1°C for $t_{d(1/2)}$ and no greater than 10% for ΔH_d.

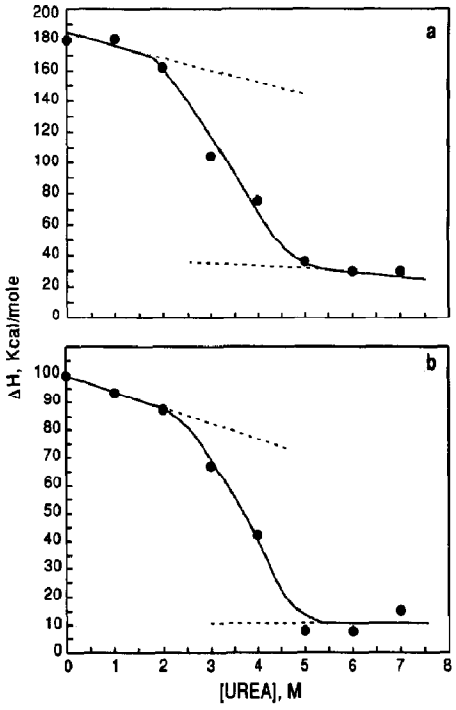

Fig. 3. The enthalpy of thermal unfolding (ΔH_d) of phycocyanins as a function of urea concentration: (a) thermophilic phycocyanin from *S. lividus*, and (b) mesophilic phycocyanin from *P. luridum*. Data were derived from Figs. 1 and 2.

753 kJ/mol for the thermophilic phycocyanin, as compared to 414 kJ/mol for the mesophilic protein. This result parallels the higher t_d for the thermophilic phycocyanin. In the presence of urea, the magnitude of ΔH_d decreases. The dependence of ΔH_d on [urea] is shown in Fig. 3. Both Fig. 3a and 3b consist of three regions representing the protein before unfolding, while undergoing unfolding, and after unfolding, respectively. The denaturation curve drops sharply between 2 and 5 M urea, and essentially levels off after 5 M urea. The [urea] at which the thermal unfolding of protein is half-completed, $(C_u)_{1/2}$, is essentially the same (3.5 M) for both proteins. In contrast, the corresponding enthalpy change at which the unfolding of protein is half-completed, $\Delta H_{d(1/2)}$, is 481 kJ/mol for thermophilic phycocyanin and 197 kJ/mol for mesophilic, which are obtained from the denaturation curve and the extrapolated straight line. Such a large difference in $\Delta H_{d(1/2)}$ between the two proteins parallels that in ΔH_d in the absence of urea.

3.2. Unfolding of thermophilic protein by urea as measured by CD

The CD spectrum of phycocyanin has a strong positive band at 630 nm and a strong negative band at 330 nm [24,25]. In the range from 240 to 199 nm, phycocyanin exhibits two shoulders, which occur at 220 and 208 nm [21–23]. To elucidate the denaturation profile of phycocyanin in urea solution, the dependence of CD (in mdeg) at 222 nm on [urea] is plotted in Figs. 4a and 4b for thermophilic and mesophilic phycocyanins, respectively. In analogy to Fig. 3, each of the figures consists of three regions representing the protein before unfolding, while undergoing unfolding, and after unfolding, respectively. The [urea] at which the protein denaturation is half-completed, $(C_u)_{1/2}$, is found to be 6.0 and 4.5 M urea for the thermophilic and mesophilic proteins, respectively (Table 2), demonstrating that the thermophilic phycocyanin is more resistant to urea denaturation than that of the mesophilic

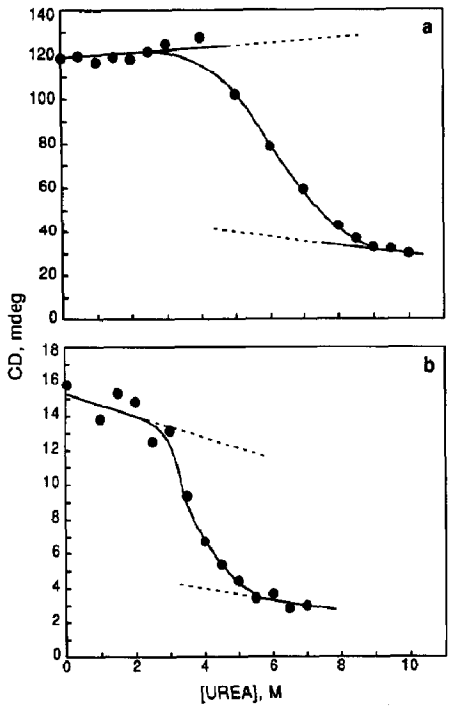

Fig. 4. Circular dichroism of phycocyanins at 222 nm as a function of urea concentration: (a) thermophilic phycocyanin from *S. lividus*, and (b) mesophilic phycocyanin from *P. luridum*. Protein concentration = 0.12 mg/ml.

Table 2
Circular dichroism and visible absorption spectrophotometric studies of the unfolding of thermophilic and mesophilic phycocyanins at 25°C

Protein	α-helix (%)	β-sheet (%)	β-turn (%)	Random coil (%)
secondary structure from CD measurements [a]				
thermophilic	52	7	20	21
mesophilic	55	3	20	22
Protein	$(C_u)_{1/2}$ (mol/l)		$\Delta G^{0'}_{[urea]=0}$ (kJ/mol)	
protein unfolding measured by CD (222 nm) [b]				
thermophilic	6.0		18.0	
mesophilic	4.5		16.7	
protein unfolding measured by visible absorption (620 nm) [b]				
thermophilic	6.5		36.4	
mesophilic	4.7		22.2	

[a] Experimental error is about ±2% for α-helix content.
[b] Experimental error is about ±0.2 mol/l for $(C_u)_{1/2}$ and ±1.3 kJ/mol for $\Delta G^{0'}_{[urea]=0}$.

one. The obtained value of 4.5 M for $(C_u)_{1/2}$ in mesophilic phycocyanin is comparable to 5.0 M as previously reported [1].

A two-state mechanism has been used to examine the denaturation of ribonuclease, lysozyme, α-chymotrypsin, lactoglobulin, and phycocyanin by denaturants such as urea or quanidine hydrochloride [1,24,27,28]. This assumption is also adopted in the present study to examine the free energy change in the unfolding of phycocyanin determined from the CD data. From data shown in Fig. 4, the equilibrium constant (K) can be determined using the following equation:

$$\text{native state} \rightarrow \text{denatured state}, \quad (1)$$

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

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 Figs. 4a and 4b. From the determined values of K, the apparent free energy of denaturation, $\Delta G^{0'}$, can be calculated according to

$$\Delta G^{0'} = -RT \ln K. \quad (3)$$

To obtain the apparent free energy of unfolding at zero denaturant concentration, $\Delta G^{0'}_{[urea]=0}$, data of $\Delta G^{0'}$ were fitted to the equation

$$\Delta G^{0'} = \Delta G^{0'}_{[urea]=0} - m[\text{urea}], \quad (4)$$

where m is a constant. Values of m range from 0.8 to 1.0 for thermophilic and mesophilic phycocyanins, comparable to 1.1 for ribonuclease and lysozyme [27], where a two-state mechanism is also adopted. Least-squares fitting of $\Delta G^{0'}$ and [urea] gives values of $\Delta G^{0'}_{[urea]=0}$ as 18.0 and 16.7 kJ/mol for thermophilic and mesophilic phycocyanins, respectively (Table 2). The obtained value of 16.7 kJ/mol for mesophilic phycocyanin is comparable to 19.3 kJ/mol as previously reported [1].

The secondary structure of thermophilic and mesophilic phycocyanins, as determined from the CD data ranging from 232 to 198 nm, is also listed in Table 2, which shows that the α-helix content in phycocyanins is about 50%, in agreement with our previously reported data for several phycocyanins [1]. In the table, no significant differences in the percentages of α-helix, β-sheet, β-turn, and random coil are found between the two proteins.

3.3. Unfolding of the protein by urea as measured by visible absorption changes

The unfolding of both thermophilic and mesophilic phycocyanins in the presence of urea was also measured using visible absorption data at 620 nm (the maximum absorption wavelength) in a similar manner to the CD experiments. In the presence of 1–10 M urea, the absorbance decreases as [urea] increases. Plots of absorbance versus [urea] are presented in Figs. 5a and 5b for thermophilic and mesophilic phycocyanins, respectively. Comparison of the denaturation curves reveals that the denaturation of the mesophile is essentially completed at the urea concentration that the thermophile denaturation begins. From these plots, values of $(C_u)_{1/2}$ were found to be 6.5

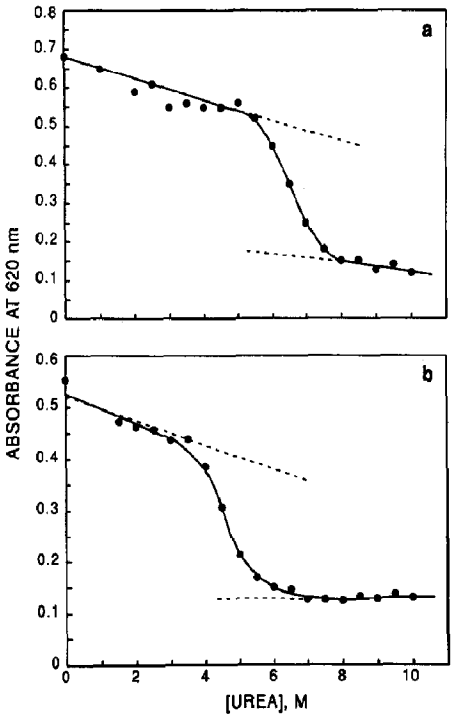

Fig. 5. The visible absorption maximum of phycocyanins at 620 nm as a function of urea concentration: (a) thermophilic phycocyanin from *S. lividus*, and (b) mesophilic phycocyanin from *P. luridum*. Protein concentration = 0.16 mg/ml.

and 4.7 M (Table 2) for thermophilic and mesophilic phycocyanins, respectively (These results are comparable to those obtained with CD.) Moreover, data of $\Delta G^{0\prime}_{[\text{urea}]=0}$ can also be found in Table 2. Its value for thermophilic phycocyanin (36.4 kJ/mol) is higher than that for mesophilic one (22.2 kJ/mol). These results are in contrast to those obtained with CD which show no significant difference in the value of $\Delta G^{0\prime}_{[\text{urea}]=0}$ between the two proteins.

4. Discussion

The two phycocyanins under comparison were isolated from two different strains of cyanophyta which were grown in the laboratory in distinct temperature environments (52 versus 25°C), and are normally found in their natural habitats growing at similar temperatures. The amino-acid compositions of phycocyanins from various strains have been found to be very similar with only minor differences [1,15,16]. These minor differences between thermophilic and mesophilic phycocyanins exhibit in both polar and nonpolar amino acid residues. Nonpolar (hydrophobic) residues are important in contributing to the difference in the free energy between the proteins [1]. These characteristics make phycocyanin a good model protein in the investigation of the nature of the structural stability for thermophilic proteins. In addition, the unfolding of phycocyanin can be monitored by both CD at 222 nm and visible absorption techniques, since phycocyanins are complex assemblies of apoproteins (polypeptides) and covalently linked chromophores (tetrapyrroles or bilins).

Table 2 shows that the value of $(C_u)_{1/2}$ in thermophilic phycocyanin, as determined by CD, is higher (6.0 M) than that in the mesophilic one (4.5 M), indicating that a higher [urea] is required to unfold thermophilic phycocyanin from helical to coil conformation than for the mesophilic one. Comparable results (6.5 versus 4.7 M) are found for the unfolding using the chromophore part of the protein to detect the change (the chromophore structure changes from a linear to cyclic conformation as the protein denatures).

Examinations of the unfolding data as presented in Table 2 reveal that the magnitude of $\Delta G^{0\prime}_{[\text{urea}]=0}$ for the unfolding is essentially the same for thermophilic and mesophilic phycocyanins (18.0 versus 16.7 kJ/mol). In contrast, the unfolding of the protein as detected from chromophore absorption changes is higher for thermophilic phycocyanin (36.4 kJ/mol), as compared with 22.2 kJ/mol for mesophilic phycocyanin. The difference in free energy as examined by the visible absorption data indicates a moderate free energy difference between the two proteins. It is suggested that the visible absorption due to perturbation of the chromophore structure is more sensitive than polypeptide structural changes as seen in the CD experiments. Since the chromophores are covalently linked to the protein at all times, it is assumed that the visible absorption experiments are sensitive to changes that are not measured by the changes in the CD at 222 nm. It is quite conceivable that significant changes in

peptide structure take place that-are not reflected in changes of the CD at 222 nm. Our finding is comparable to the observation that the free energy of stabilization of thermophilic proteins is 20–40 kJ/mol larger than that of corresponding mesophilic proteins [5,6].

Results of differential scanning calorimetry as shown in Table 1 and Fig. 3 show that the magnitude of $(C_u)_{1/2}$, at which the thermal unfolding of the protein is half-completed, is essentially the same (3.4 versus 3.6 M of urea) for thermophilic and mesophilic proteins. To accommodate comparable values of $(C_u)_{1/2}$, the corresponding $(t_d)_{1/2}$ and $(\Delta H_d)_{1/2}$ are much higher for the thermophilic than the mesophilic protein (65 versus 48°C, and 481 versus 197 kJ/mol, respectively). In other words, at comparable values of $(C_u)_{1/2}$, much higher temperature and enthalpy of unfolding are observed for the thermophilic protein than for the mesophilic one. These observations parallel those in the absence of urea, where $\Delta H_d = 753$ kJ/mol for thermophilic phycocyanin and 414 kJ/mol for mesophilic protein. Such an intrinsic thermal stability for a thermophile has also been shown for D-glyceraldehyde-3-phosphate dehydrogenase from a thermophilic bacterium [29].

The observations of a large difference in the enthalpy of unfolding in contrast to a moderate difference in the free energy of unfolding suggest that a significant entropy change accompanies the unfolding of phycocyanins. A much higher entropy contribution is observed for the thermophilic one, due to its much larger ΔH_d. This higher entropy of unfolding in the thermophilic protein is believed to derive from a more rigid overall structure of protein rather than a higher helical content, since the percentage of α-helix is essentially the same for the thermophilic and mesophilic proteins (Table 2).

The more rigid structure in the thermophilic protein should lead to more resistance to protein denaturation by an external parameter such as temperature or the nature of the solvent. Our results confirm that the thermophilic protein is more impervious than the mesophilic one to reagents such as urea, which is a structure breaker and is thought to disrupt hydrophobic interactions [30,31]. The disruption of hydrophobic interaction is usually associated with a large change in the entropy. The stronger hydrophobic interaction in the apoprotein part of thermophilic phycocyanin is expected to play an important role in its higher structural stability over the mesophilic one. In engineering proteins with enhanced thermostability, substitutions are made that increase internal hydrophobicity, that increase hydrophobicity in α-helical regions [3,4], and that stabilize helices for strong internal packing [13,14]. In addition, the multiple amino acid replacements in thermophilic proteins also cause increased external polarity [9,11].

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