The Free Energy of Dissociation of Oligomeric Structure in Phycocyanin Is Not Linear with Denaturant†

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ABSTRACT: Using SEC HPLC and fluorescence anisotropy, absorption spectra were assigned to the specific oligomeric structures found with phycocyanin. The absorption spectra were used to quantify the population of each oligomeric form of the protein as a function of both urea concentration and temperature. Phycocyanin hexamers dissociate to trimers with equilibrium constants of 10^{-6} to 10^{-5}. Phycocyanin trimers dissociate to monomers with equilibrium constants of 10^{-15} to 10^{-12}. Both dissociation constants increase linearly with increasing urea concentration, and $\Delta G°$ values calculated from the equilibrium constants fit best with an exponential function. Our findings appear in contrast with the commonly used linear extrapolation model, $\Delta G_{urea}° = \Delta G_{water}° + A$[denaturant], in which a linear relationship exists between the free energy of protein unfolding or loss of quaternary structure and the denaturant concentration. Our data examines a smaller range of denaturant concentration than generally used, which might partially explain the inconsistency.

Many active biological complexes are formed from the association of small subunits. While large proteins should be more stable due to their increased ratio of buried to solvent-exposed surface area, a multidomain protein folds more slowly than a single domain protein (*1*−*3*). Thus, there appear to be some advantages to building active complexes through small, single domain subunits. There is a broad and detailed body of knowledge on both the kinetics and thermodynamics of single domain folding, but the field of knowledge for the association or oligomerization process is more limited.

In this paper we examine the thermodynamics of the urea induced dissociation of the oligomeric structures in phycocyanin. Phycocyanin is a chromoprotein found in the light harvesting antenna or phycobilisome of cyanobacteria (*4*, *5*). The basic unit of phycocyanin is a heterodimeric $\alpha\beta$; the 17.4 kDa α subunit contains one covalently bound chromophore while the 17.4 kDa β subunit contains two covalently bound chromophores. The chromophore is a conjugated tetrapyrrole system known as the phycocyanobilin. There is a large degree of homology between the α and β subunits with each subunit having an all-helical globin-like structure. The protein used in this study has been isolated from *Agmenellum quadruplicatum*, and the crystal structure of hexameric phycocyanin isolated from this species has been determined (*6*, *7*).

Phycocyanin has well-defined quaternary forms, each with a unique spectroscopic signature (*5*, *8*). The largest complex examined was a hexamer of heterodimers, $(\alpha\beta)_6$. Upon the addition of urea, the hexamer dissociates into trimers, $(\alpha\beta)_3$, monomers, $(\alpha\beta)$, and finally into the isolated, unfolded α and β subunits. The absorption and fluorescence spectra of the chromophores are sensitive to the environment of the chromophore binding pocket and, thus, respond to changes in the oligomeric structure which induce changes in the binding pocket (*5*, *9*). With careful selection of buffer conditions, we have been able to observe transitions between each oligomeric state of the protein, and we monitor the dissociation using the absorption of the covalently attached tetrapyrrole chromophores. The hexamer is closest to the "active" structure; it is virtually indistinguishable spectroscopically from phycocyanin in the antenna complex.

There are three different surfaces that are buried upon formation of the oligomeric structure (*6*). First, the interface between the α and β subunits is buried upon formation of the heterodimer (commonly called the monomer form in the literature). Second, three monomers can assemble into a disk-like trimer which buries surface area between the α subunit on one monomer and the β subunit on a second monomer. Third, hexamers are made from the head to head association of trimers (*6*).

We chose phycocyanin as our model system based upon three factors. First, phycocyanin is a fairly typical all-helical protein (*6*), and it could serve as a good model for many water-soluble systems. Second, we can use either fluorescence or absorption spectroscopy to follow the dissociation reaction. Many techniques commonly used to monitor protein folding such as CD or intrinsic fluorescence do not have

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appreciable signals for oligomerization reactions. The hexamer form of phycocyanin has both large extinction coefficients and quantum yields, and both properties decrease with the loss of oligomeric structure. Therefore, the spectral changes that occur upon dissociation are large with excellent signal to noise. Third, we have developed solvent conditions that appear to give well-defined oligomeric states with specific urea concentrations.

We will use our system to test the assumption that the linear approximation, $\Delta G°_{urea} = \Delta G°_{water} + A[\text{urea}]$, can be used to model the dissociation of oligomeric structure (*10*, *11*) in a way similar to its use for the loss of tertiary structure (*12*−*14*). In a second set of experiments, we quantified the concentrations of each oligomeric form as a function of both temperature and urea concentration to get the free energy and equilibrium constants for hexamer dissociation. It is beyond the scope of this paper to provide a complete mathematical model to describe the dissociation of oligomeric structure.

MATERIALS AND METHODS

All chemicals used were purchased from Sigma-Aldrich and were of reagent grade. Generally, urea stock solutions were freshly prepared to eliminate problems from urea degradation in solution.

Cell Growth. A culture of *Synechococcus sp PCC* 7002 (*Agmenellum quadruplicatum*) was obtained from the American Type Culture Collection. The culture was maintained as a continuous liquid culture using ambient light. Larger quantities of cells were grown in 2.5 L low form flasks with continuous stirring at room temperature with the addition of approximately 3% CO_2/N_2 gas in the air space above the medium. Additional light was provided using commercial growth lights. Cells were harvested, resuspended in 50 mM sodium acetate, pH 5.0, and stored at −20 °C until needed.

Isolation of Phycocyanin. The protein was isolated from cells according to a procedure described by Glazer and Fang (*15*) with the following modification. The cells were lysed with a Bio-Neb nebulizer in three cycles using 100 psi of N_2 gas, rather than using a French press. The phycocyanin fractions recovered from the final DEAE column were combined, precipitated by addition of ammonium sulfate to 65% saturation, and stored as the precipitate at 4 °C until required. Stock solutions of protein (typically 300 μM) were made by resuspending the precipitate in a small amount of 5 mM potassium phosphate, pH 7.0. The concentration of the stock was determined using $\epsilon_{663} = 106\,500\ M^{-1}\ cm^{-1}$ in 8.0 M urea, pH 3.0 (*15*).

Isothermal Spectral Data. Individual solutions of phycocyanin were prepared with varying concentrations of urea in 0.50 M potassium phosphate buffer, pH 8.0. The solutions were allowed to equilibrate at 293 K overnight. All steady-state spectra were taken using a Varian Cary 300 UV−vis spectrometer with a thermostatted cuvette holder at 293 K.

Temperature Studies. Solutions of phycocyanin in the appropriate urea/buffer solution were prepared at least 2 h prior to measurement. We found no difference between data acquired with 1.5 h versus 24 h equilibration times. After the initial equilibration time, the sample was pipetted into a quartz cuvette and placed into the thermostatted cuvette holder of the Varian Cary 300 UV−vis spectrometer. The temperature of the circulating water bath was changed to control the temperature of the sample. The sample was allowed to equilibrate at each temperature (which ranged from approximately 278 to 308 K), and the spectrum was obtained after no further changes in the spectrum occurred. Generally, samples equilibrated in 6 to 8 min. No turbidity was detected in the samples during the course of the temperature studies. Samples containing guanidine hydrochloride were examined in a similar way except the equilibration time for the samples was approximately twice as long with this denaturant.

Characterization of Oligomeric Structure with HPLC. Assignment of the monomer and α/β structures was confirmed by size exclusion HPLC with a Tricorn Superdex 200 10/300 GL column (Amersham Biosciences). The calibration curve was created with standards of BSA, cytochrome *c*, and vitamin B_{12} in the same solvent. Samples were run at room temperature with a flow rate of 0.5 mL/min and pressures typically around 70 psi. The standards were detected by 280 nm absorption. The phycocyanin was detected by 620 nm absorption. An Ocean Optics UV−vis with CCD detection (model ISS-UV-Vis) was used to obtain absorption spectra immediately after elution from the column.

Acquisition of Reference Absorption Spectra for the Oligomeric States. The solvent conditions used to obtain "pure" oligomeric forms were as follows: hexamer solutions contained 0.75 M potassium phosphate, pH 7.0, the trimer solutions contained 0.010 M potassium phosphate, pH 8.0, and the monomer solutions contained 1.0 M NaBr, 0.10 M sodium acetate, pH 5.5. Standard spectra for the monomer were also collected with urea (from 0.30 to 3.0 M) to account for broadening due to the change in dielectric of the solution. Four identical samples of 2.0 μM phycocyanin (concentration in monomer) were prepared in that solvent and allowed to equilibrate. The spectra were acquired, averaged, and divided by the protein concentration to give the reference spectrum for that specific oligomeric form. All data were taken at 298 K. Only two spectra were averaged for the monomer standards in urea.

Anisotropy Measurements. The monomer samples were made with 4.0 μM phycocyanin, 1.0 M NaBr, 0.50 M potassium phosphate, pH 8 with the addition of ethylene glycol (up to 3 M) to increase the viscosity. The trimer samples were made with 4.0 μM phycocyanin, 0.010 M Hepes, pH 8.0 with the addition of glycerol (up to 3 M) to increase the viscosity. The hexamer samples were made with 4.0 μM phycocyanin, 0.50 M potassium phosphate, pH 7.0 with the addition of glycerol (up to 0.8 M) to increase the viscosity. The samples that contained a mixture of hexamer and trimer were made with 0.30 M urea along with 0.5 M potassium phosphate, pH 8.0. All samples were equilibrated at 293 K for least 3 h prior to measurement. The anisotropy values were obtained on a Perkin-Elmer LS 50B fluorimeter with an excitation wavelength of 620 nm, an emission wavelength of 650 nm, 7.5 nm slits, and at least 15 s of averaging time per measurement. The fluorimeter was equipped with a thermostatted cuvette kept at 293 K. All measurements were acquired using a quartz microcuvette. The viscosity of urea solutions was estimated using the data given in Kwahara and Tanford (*16*). The viscosity of the glycerol, ethylene glycol, and potassium phosphate solu-

tions was estimated from data given in the CRC handbook (*17*).

Calculating Concentrations from Absorption Spectra Acquired in Temperature Studies. All data were imported into Igor Pro. The spectrum of the solution at each temperature was assumed to be a linear combination of hexamer, trimer, and monomer contributions. Contributions from each species were found using the following function: raw spectrum = $c_1 \times$ hexamer reference spectrum + $c_2 \times$ trimer reference spectrum + $c_3 \times$ monomer reference spectrum. No restrictions were put on the constants, c_1, c_2, c_3, except that they were not allowed to be negative. We then converted the constants to concentrations of each oligomeric form and calculated equilibrium constants for each dissociation reaction. From the equilibrium constants, we were able to calculate the free energies .

RESULTS

Absorption of Phycocyanin as a Function of Urea. The absorption spectrum of phycocyanin is exquisitely sensitive to urea concentration. In Figure 1A, we show the absorption spectra of 2.0 μM phycocyanin as a function of urea concentration at 293 K. The samples were incubated overnight, which according to our controls should be more than sufficient for equilibration. We found that our samples equilibrated in the urea solutions in less than an hour with no further changes in the next 20 h, as monitored by UV−vis absorption spectroscopy. Data from control experiments are shown in the Supporting Information.

In the absence of urea, the spectrum has an absorption maximum at 627 nm with a shoulder at 600 nm. As the concentration of the denaturant is increased, both the molar absorptivity and the absorption maximum decrease. If we plot the ratio of (A_{627}/integrated area of absorption spectrum) versus the urea concentration as in Figure 1B, we find three significant transition points in our data: a sharp decrease at approximately 0.1 M urea, a second decrease at approximately 1.1 M urea, and a third transition at 5.4 M. We completed these experiments with both 2.0 and 4.0 μM phycocyanin; the transitions are independent of the protein concentration.

Measurement of Reversibility of Transitions. The first two transitions shown in Figure 1B appear to be reversible upon dilution of the denaturant. Since the chromoprotein is part of a light harvesting structure in cyanobacteria, the best way to quantify the activity of the protein is to measure its quantum yield. We equilibrated the protein in 0.50 M urea and 1.5 M urea in the buffer conditions described above for 4 h. Using 0.50 M potassium phosphate buffer, we diluted out the urea to 0.050 and 0.60 M, respectively. The samples were left at 293 K overnight to equilibrate. We found that samples had the same quantum yield (within 4%) as their controls, solutions which contained the same amount of urea as the diluted samples and were made at the same time and temperature. We also found that when we incubated the protein for several hours in 6.0 M urea with 0.50 M potassium phosphate, we lost significant amounts of the protein to precipitation when we tried to refold the protein by diluting the urea to 3.0 M.

Characterizing the Absorption Spectrum of Each Oligomeric Structure. We attempted to confirm all assignments

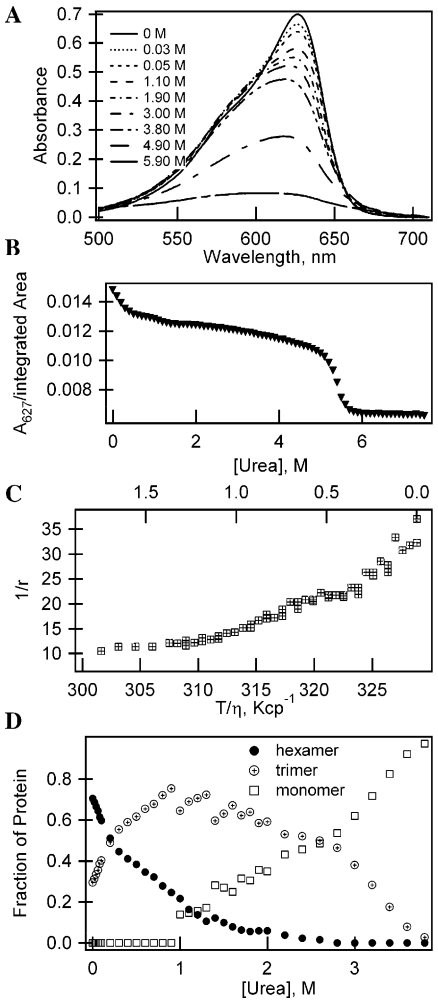

FIGURE 1: Changes in phycocyanin as a function of urea concentration. All samples contain 0.50 M potassium phosphate, pH 8.0 along with the urea and were equilibrated at room temperature overnight. Spectra were taken at 293 K. (A) Absorption of 2.0 μM protein. (B) Ratio of absorbance at 627 nm over integrated peak area plotted versus urea concentration for 4.0 μM protein. (C) Perrin plot as a function of urea with 4.0 μM protein. Data taken with 620 nm excitation, 650 nm emission, and 8.5 nm slits. (D) Oligomeric concentrations obtained from linear deconvolution of absorption spectra.

with SEC HPLC. The solvent conditions used to obtain homogeneous populations of the oligomeric structures were selected based upon a close reading of the literature for phycocyanin from a variety of species including *Anacystis nidulans*, *Anabaena variabilis*, and *Plectonema calothricoides* (8, *18*−*25*). Most of the oligomeric structures identified in the literature were determined using ultracentrifugation sedimentation. Only the smaller forms − the α and β subunits and the monomer − gave unambiguous peaks on the HPLC column. Phycocyanin in 1.0 M NaBr, 0.10 M sodium acetate at pH 5.5 gives a single peak with an elution time corresponding to the mass of the monomer (34 kDa). We were also able to get a single peak corresponding to the mixture of denatured α and β subunits in 5.0 M urea, 0.50 M potassium phosphate, pH 8.0; each subunit is 17.4 kDa. Attempts to isolate homogeneous elution bands for either the trimer or the hexamer forms of the protein were unsuccessful; the protein eluted as a mixture. We see no evidence for any oligomeric state larger than a hexamer. We

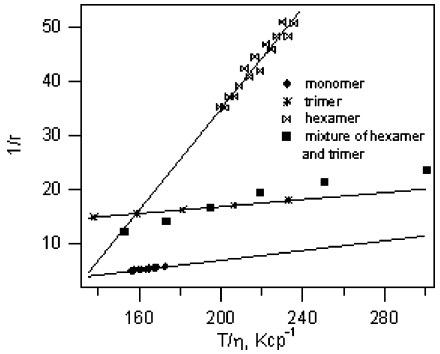

FIGURE 2: Perrin plots for specific oligomeric forms of phycocyanin. The monomer samples were made with 4.0 μM phycocyanin, 1.0 M NaBr, 0.50 M potassium phosphate, pH 8 with the addition of ethylene glycol (up to 3 M) to increase the viscosity. The trimer samples were made with 4.0 μM phycocyanin, 0.010 M Hepes, pH 8.0 with the addition of glycerol (up to 3 M) to increase the viscosity. The hexamer samples were made with 4.0 μM phycocyanin, 0.50 M potassium phosphate, pH 7.0 with the addition of glycerol (up to 0.8 M) to increase the viscosity. The samples that contained the mixture of states had 0.30 M urea with 0.50 M potassium phosphate, pH 8.0. All samples were equilibrated at 293 K for at least 3 h prior to measurement. The anisotropy was measured as described in Figure 1C. Least-squares analysis gave correlation coefficients of 0.999 for the trimer and monomer and 0.98 for the hexamer data.

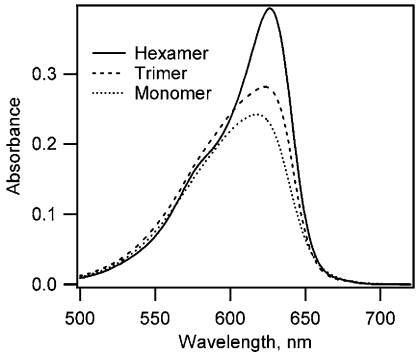

FIGURE 3: Reference absorption spectra. Each spectrum is of 1.00 μM (in monomer) phycocyanin in the given oligomeric form. The solvent conditions are as follows: hexamer, 0.75 M potassium phosphate, pH 7.0; trimer, 0.010 M potassium phosphate, pH 8.0; monomer, 1.00 M NaBr, 0.100 M sodium acetate, pH 5.5, and all solutions at 298 K.

monitored the absorption spectrum of the protein as it eluted off the column; as the elution time increased, the absorption maximum of the protein shifts further to the blue, data shown in the Supporting Information.

Since the oligomeric structure of phycocyanin appears to be sensitive to the HPLC column conditions, we used fluorescence anisotropy to characterize the absorption spectrum of each phycocyanin oligomer. All fluorescence anisotropy data are plotted in the form of the Perrin equation, eq 1, in which r is the measured anisotropy, r_o is the limiting anisotropy, k is Boltzmann's constant, τ_F is the fluorescent lifetime, V_h is the hydrated volume of the protein, T is the temperature in K, and η is the viscosity of the solution (*26*). In Figure 1C, we have plotted fluorescence

$$1/r = 1/r_o + \tau_F kT/V_h\eta \quad (1)$$

anisotropy data from phycocyanin using urea both to vary the viscosity of the solution and to act as a denaturant. The viscosities of the urea solutions were calculated using the published values (*16*). Two transitions are readily apparent in the Perrin plot at T/η of approximately 325 K cP^{-1} (approximately 0.25 M urea) and T/η of approximately 313 K cP^{-1} (approximately 1 M urea). The anisotropy is 0.02 at 0 M urea, and this value does correspond to the published value for the hexamer form of the protein at similar excitation and emission wavelengths (*27*, *28*).

To show that we had homogeneous populations associated with a specific absorption spectrum, we prepared Perrin plots for phycocyanin in solvents which according to previously published studies should result in a homogeneous population of hexamer, trimer, or monomer, Figure 2. Monomer samples were made in 1.0 M NaBr, 0.50 M potassium phosphate buffer, pH 8.0, and the viscosity of the solution was increased with the addition of ethylene glycol. After the samples equilibrated at 293 K, the absorption spectra and the fluorescence anisotropy were measured. We saw no changes in our absorption spectrum due to the addition of the ethylene glycol; not only did our absorption spectra overlay for the nine samples, but they also matched the absorption spectrum assigned to the monomer form in Figure 3. In addition, we had a linear Perrin plot; the hydrated volume of the protein is not changing with the addition of the ethylene glycol. The monomer is the only stable oligomeric form in 1.0 M NaBr, and we confirm the SEC HPLC assignment of the absorption spectrum shown in Figure 3 to the monomer. We obtained identical results for phycocyanin in 0.010 M Hepes, pH 8.0 with the addition of glycerol to increase the viscosity at 293 K. According to the literature, phycocyanin should be a stable trimer under solvent conditions of low salt and high pH (*8*). The absorption spectra were unchanged with the addition of glycerol, up to 3.0 M, and identical to the absorption spectrum in Figure 3.

According to the literature, the hexamer is generally found in high salt buffer at neutral or low pH (*8*). We equilibrated phycocyanin at 293 K in 0.50 M potassium phosphate buffer at pH 7.0 with the addition of glycerol to increase the viscosity and acquired fluorescence anisotropy and absorption spectra data. There was a negligible change in the absorption spectrum with the addition of the glycerol which may be due to the change in solvent dielectric; the absorption spectra are shown in the Supporting Information and are virtually identical to the absorption spectrum assigned to the hexamer shown in Figure 3. The Perrin plot, Figure 2, was linear, indicating a homogeneous population.

Last, to check the sensitivity of the method, we equilibrated phycocyanin in 0.50 M potassium phosphate buffer at pH 8.0 with 0.30 M urea and added glycerol to increase the viscosity; the addition of the urea should create a mixture of both hexamer and trimer according to our data. As seen in Figure 2, we found that the Perrin plot was curved, and the absorption spectra no longer overlaid; the absorption spectra maxima shifted to the blue with increasing glycerol concentration, indicating a change in the oligomeric structure, data shown in the Supporting Information. We can distinguish changes in oligomeric forms using fluorescence anisotropy and the absorption spectroscopy.

Deconvoluting Absorption Spectra To Get Individual Contributions. Using the absorption spectra in Figure 3 as

our reference spectra, we found the concentration of each oligomeric state at 293 K as a function of urea concentration. We calculated the contribution of each oligomeric form (hexamer, trimer, and monomer) to the raw absorption spectrum by assuming that the spectrum is a linear combination of the absorption of the three forms, and that each form is accurately represented by the spectra given in Figure 3. No restrictions were put on the fit except that contributions could not be negative. The result is shown in Figure 1D. We initially had problems fitting spectra at urea concentration higher than 1 M. At that point, the quality of the fit suffered due to a broadening of the raw spectrum; we attribute this effect to the change in the dielectric of the solvent rather than to changes in the oligomeric structure. We were relatively successful in bypassing this effect up to 3.0 M urea by using a reference spectrum for the monomer that contained the same amount of urea as the raw spectrum (to within 0.1 M). We measured an entire set of reference spectra for the monomer that included urea from 0 M urea to 3.0 M urea in 0.3 M steps along with the 1.0 M NaBr, 0.10 M sodium acetate, pH 5.5. SEC HPLC confirmed that phycocyanin was still a monomer (>90% monomer, remainder α and β subunits) up to 3.0 M urea in the presence of the 1.0 M NaBr and 0.10 M sodium acetate, pH 5.5.

Our method to quantify the concentrations appears to work well at low and high urea concentrations where two states are mainly present. The signal to noise slightly suffers when all three forms are present, from approximately 1 M urea to 2 M urea. We find that we do not start with a pure solution of hexamer at 0.0 M urea; the solution contains 75% of the protein in the hexamer and 25% in the trimer. The hexamer concentration rapidly decreases with increasing urea, but it appears to persist up to 1.5 M urea. The trimer concentration rapidly increases with the increasing urea concentration, levels off, and then starts to fall at approximately 2 M urea. We did not find any trimer in concentrations greater than 3.8 M urea. We did not find any monomer present in the solution at 293 K until the urea concentration was greater than 0.9 M. Its concentration increases with increasing urea until it is the only species left in the solution at urea concentrations greater than 3.8 M. The transition seen in Figure 1B at approximately 0.1 M urea appears to correlate with the point where the protein is equally distributed between the hexamer and trimer forms. The transition seen at approximately 1.1 M urea roughly correlates with the point in which the monomer starts to appear.

Temperature Studies on the Hexamer and Trimer Transitions. We examined the hexamer and trimer dissociation as a function of temperature and urea concentration. An example of the temperature sensitivity is shown by the changes in the absorption spectra for phycocyanin in 0 M urea, 0.50 M potassium phosphate, pH 8.0, Figure 4A. Above 312 K, we appear to have some irreversible loss of protein, but the changes in the absorption spectrum below 308 K are reversible with temperature. We ran a series of controls at various protein and urea concentrations to ensure that the protein had reached equilibrium with each small temperature change; judging from changes in the absorption spectrum, the protein appears to equilibrate to a new temperature in less than 6 min with no further changes over the next 2 or 3 h. An example of our temperature control is included in the Supporting Information.

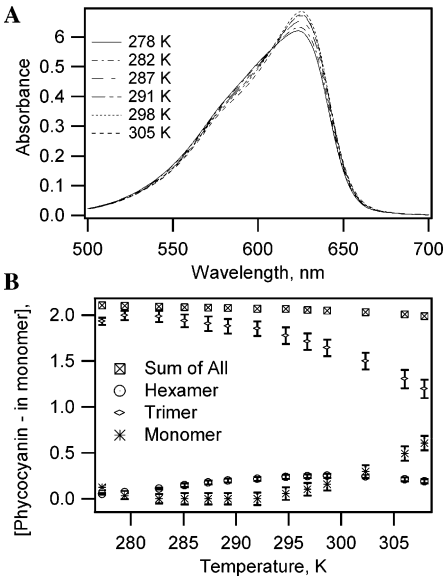

FIGURE 4: Temperature dependence of quaternary structure. Sample contains 2.0 μM (in monomer) phycocyanin in 0.50 M potassium phosphate, pH 8.0, and was equilibrated at room temperature for 3 h and at 278 K for 1 h prior to measurement. The sample was allowed to equilibrate at least 8 min at each temperature. (A) Representative absorption spectra. (B) Representative concentrations calculated from absorption spectra taken with samples containing 0.80 M urea. Errors bars represent 95% confidence interval as calculated by Igor Pro.

We calculated the contributions of each oligomeric form (hexamer, trimer, and monomer) to the absorption spectrum assuming it is a linear combination of the absorption of the three forms of the protein; typical data for a single trial are shown in Figure 4B. We also calculated the total protein content in each sample by adding our individual concentrations, as denoted by the "Sum of All". The concentrations calculated using this method are remarkably consistent with the total concentration ending up very close to the true concentration (as measured from the protein stock solution). We do see a slight decrease, less than 4%, in the total protein content over the course of the experiment, which we attribute to an irreversible denaturation reaction at high temperatures; longer times spent at the higher temperatures led to a larger decrease. An example of some raw data with its best fit is given in the Supporting Information.

Since we have the equilibrium concentrations for hexamer, trimer, and monomer at a number of temperatures and denaturant concentrations, we can calculate equilibrium constants for each dissociation reaction. As a control, we acquired data for protein concentrations from 1 μM to 8 μM phycocyanin in 0.50 M potassium phosphate pH 8.0 with no urea present. We then compared the equilibrium constants for the dissociation of the hexamer at 293 K for each protein concentration. The equilibrium constants ranged from 2×10^{-7} with 4 μM phycocyanin to 7×10^{-7} with 1 μM phycocyanin with an average of 4 ($\pm$2) $\times 10^{-7}$ for the entire range. No systematic trends were noted except that the equilibrium constants did appear to be related to specific preparations of the protein, i.e., some preparations gave higher equilibrium constants while other preparations gave lower numbers. The variation may correlate with the amount of time that the protein is stored as an ammonium sulfate precipitate, our method of long-term protein storage. The

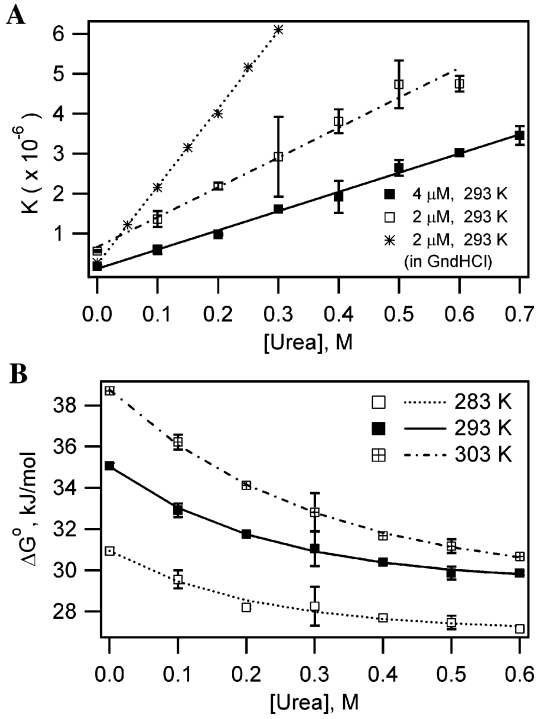

FIGURE 5: The dissociation of the hexamer. The samples are prepared as described in Figure 4 with the addition of urea or guanidine hydrochloride and either 2.0 or 4.0 μM phycocyanin (in monomer). (A) The equilibrium constants for the dissociation reaction with the best linear fit to the data obtained by least-squares analysis as denoted by the lines. Error bars were calculated from the standard deviations calculated from averaged data. Each data point is an average of at least three samples. All correlation coefficients are at least 0.99. (B) Free energy of hexamer dissociation. The solid line is the best single-exponential fit, $y = y_o + C\,e^{-kx}$. The best fits shown have the following constants of y_o, C, and k: at 283 K, 26.1($\pm$0.3) kJ/mol, 3.9($\pm$0.3) kJ/mol, 4.8($\pm$1) M^{-1}; at 293 K, 28.4($\pm$0.2) kJ/mol, 5.6($\pm$0.2) kJ/mol, 4.4($\pm$0.4) M^{-1}; at 303 K, 28.3($\pm$0.3) kJ/ mol, 8.6($\pm$0.3) kJ/mol, 3.5(0.3) M^{-1}.

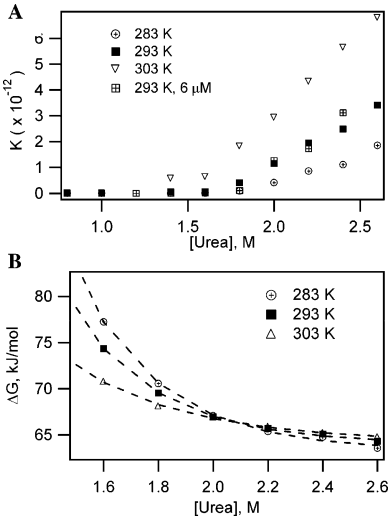

FIGURE 6: The dissociation of the trimer. The samples are prepared as described in Figure 4 with the addition of urea and 4.0 or 6.0 μM phycocyanin (in monomer). (A) Equilibrium constants with each data point for the 4.0 μM protein an average of at least three samples while the 6.0 μM samples are individual samples. (B) Free energy of trimer dissociation as a function of urea and temperature. The free energy was calculated from the equilibrium constants given in panel A (above 1.5 M urea only). The dotted line is the best single-exponential fit, $y = y_o + C\,e^{-kx}$.

equilibrium constants shown below are an average of samples made from multiple preparations.

The equilibrium constants obtained typically ranged from 10^{-5} to 10^{-7} for the dissociation of hexamer and 10^{-13} to 10^{-17} for the dissociation of the trimer; both equilibrium constants increased with increasing urea concentration. The equilibrium constants decreased with temperature for the hexamer dissociation, but increased with temperature for the trimer dissociation. Averaged equilibrium constants for the hexamer dissociation at 293 K are shown in Figure 5A for two protein concentrations (2.0 and 4.0 μM phycocyanin) and two types of denaturants (urea and guanidine hydrochloride); each point is an average of at least three separate trials. The equilibrium constants for the trimer dissociation are shown in Figure 6A for two protein concentrations, 4.0 and 6.0 μM phycocyanin. Our equilibrium constants agree within an order of magnitude with earlier measurements made using ultracentrifugation at 294 K under different solvent conditions (*8*).

Perhaps the most remarkable feature noted for the hexamer dissociation equilibrium constants, Figure 5A, is that they appear to increase linearly with denaturant concentration. The trimer dissociation equilibrium appears to follow a similar linear trend but only above urea concentrations of 1.5 M, Figure 6A. The effect of protein concentration is hard to discern from our data; in the trimer dissociation the data acquired with 4.0 and 6.0 μM phycocyanin coincide well, but there does appear to be a systematic difference in the hexamer dissociation. In both cases, we examined a limited range of denaturant concentrations.

We calculated the free energy for hexamer dissociation at each urea concentration using the equilibrium constants, and a subset of our data for 2 μM phycocyanin is shown in Figure 5B. The $\Delta G°$ drops sharply with the initial additions of urea, but it levels out to a relatively constant value by 0.60 M urea. The initial decrease in $\Delta G°$ appears to be steeper with higher temperatures. As seen with the equilibrium constants, the dissociation of the hexamer appears to be less spontaneous at 303 K compared to 283 K. The free energy of the trimer dissociation with 1.5 M urea and above is shown in Figure 6B. Once again, the data above 1.5 M urea fits to an exponential function, and the data levels out around 2.4 M urea. In the case of the trimer dissociation, the dissociation appears to be less spontaneous at 283 K compared to 303 K, in contrast to the hexamer. The Gibbs free energy is significantly higher for the dissociation of the trimer compared to that of the hexamer.

DISCUSSION

Relationship between the Absorption Spectrum and the Oligomeric Structure. The absorption spectrum of phycocyanin is sensitive to the amount of denaturant present in the solution. As shown in Figure 1B, we find three significant transition points in our data: a sharp decrease at approximately 0.1 M urea, a second decrease at 1.1 M urea, and a third transition at 5.4 M. The transition that is the easiest to understand and assign is the large transition at 5.4 M; it is well documented in the literature that the sharp decrease in visible absorption at high urea concentrations is due to loss of the heterodimeric unit ($\alpha\beta$) coupled with

unfolding of the tertiary structure (*15*, *21*, *29*). The tertiary structure of the protein is no longer working as a scaffold to hold the tetrapyrrole chromophore in a linear conformation, and the chromophore assumes a "lockwasher" conformation with a much smaller molar absorptivity in the visible region (*9*, *30*). Therefore, we will assign the transition at 5.4 M urea to the conversion of ($\alpha\beta$) to unfolded protein. This assignment agrees with our SEC HPLC data for high urea concentrations; the protein elutes at a time corresponding to 17−18 kDa, the mass of the individual α and β subunits.

The other two transitions apparent in Figure 1B have not been documented in the literature, but they clearly appear to be tied to the loss of higher levels of oligomeric structure since the absorption spectrum of phycocyanin is sensitive to the oligomeric structure of the protein (*31*). Three oligomeric forms are commonly found with phycobiliproteins: a hexamer of the heterodimer, $(\alpha\beta)_6$, a trimer of the heterodimer, $(\alpha\beta)_3$, and the $\alpha\beta$ heterodimer which is also called a monomer in the literature (*4*, *5*). We will use the common names for the oligomeric structures where hexamer is $(\alpha\beta)_6$, trimer is $(\alpha\beta)_3$, and monomer is ($\alpha\beta$). Logically, since the transition that occurs at 5.4 M urea is the loss of the $\alpha\beta$ heterodimer, it would appear that the transition at approximately 0.1 M urea is a transition between the hexamer and trimer forms, and the transition at 1.1 M urea is the transition between the trimer and monomer forms of the protein.

Further evidence that our spectral changes are due to changes in the oligomeric structure of phycocyanin can be seen in Figure 1C. Two transitions are readily apparent in the Perrin plot at urea concentrations of approximately 0.25 and 1 M which indicate changes in protein structure, either in the hydrated volume of the protein or the limiting anisotropy of the chromophores. The conventional interpretation of the data using the Perrin equation to obtain a hydrated volume of the protein will not suffice due to the energy transfer that will occur in the larger oligomeric structures. We observe behavior that is the opposite of what is expected for a transition in which the size of the macrostructure is decreasing, but this inconsistency is easily explained by the depolarization of the emission that will occur with energy transfer between the 18 chromophores in the hexamer and the 9 chromophores in the trimer. Clearly, from the changes in the slope, the protein and/or chromophore conformations are changing as urea is added.

The Loss of Hexamer and Trimer Structure Is Fully Reversible. From Figure 1B, phycocyanin incubated in 0.5 M urea and diluted out to 0.05 M should undergo a shift in structure which we anticipate to be a change from trimer to hexamer. The sample incubated with 1.5 M urea and diluted to 0.60 M urea is anticipated to undergo structure changes from primarily monomer to primarily trimer. From our measurements of fluorescence quantum yield, we find that urea induced transitions between the different oligomeric forms appear to be reversible with no loss of protein activity since the quantum yields were virtually unchanged. Unfortunately, it does appear that our transition from the unfolded α and β subunits at 6.0 M urea to monomer at 3.0 M urea is not totally reversible under these specific solvent conditions; therefore, the focus of this paper will be on the first two transitions.

Assignment of Spectra to Oligomeric Forms. We found that SEC HPLC was successful in determining size in only the unfolded protein (as a mixture of α and β subunits) and the monomer form, ($\alpha\beta$). Based upon the absorption of the protein as it eluted from the column, it appears that the chromatography perturbed the equilibrium between the hexamer and trimer forms. Therefore, we used fluorescence anisotropy to confirm the spectral assignments shown in Figure 3. The validity of the approach is illustrated by the Perrin plot of phycocyanin, Figure 1C, using the denaturant to increase the viscosity; the three transitions observed in Figure 1B from the absorption spectra can also be seen with the changes in slope in the Perrin plot.

We found linear Perrin plots, Figure 2, for the solvent conditions that selected for hexamer, trimer, and monomer forms of phycocyanin, according to published literature for phycocyanin from other species (*8*). In each case, we found that our absorption spectra were unchanged with the solvent conditions used to increase the viscosity of the solution. A linear Perrin plot indicates that the hydrated volume of the protein complex is not changing with the addition of either ethylene glycol or glycerol, and our buffer conditions strongly favor one oligomeric form. We tested the sensitivity of the method by adding 0.30 M urea to create a mixture of hexamer and trimer, and we found not only that the absorption spectrum became very sensitive to the addition of the glycerol but also that the Perrin plot was curved. The fluorescence anisotropy and absorption data give consistent results, and they validate the spectral assignments given in Figure 3.

Urea Dependence of the Oligomeric Structures. We used linear deconvolution of the absorption spectra shown in Figure 1A to quantify the amount of each oligomeric state present in the equilibrated solution at 293 K, Figure 1D. The amount of hexamer rapidly falls with increasing urea concentration, but it is present in low concentrations past 2.0 M urea where there are still substantial amounts of trimer present in the solution. The first transition seen in Figure 1B reflects the transition between the hexamer and trimer; the midpoint of the transition appears to coincide with the urea concentration in which equal amounts of phycocyanin are tied up in the hexamer and trimer species. The second transition seen in Figure 1B is harder to interpret since all three oligomeric forms are present in substantial amounts at this point. Based upon the concentration data shown in Figure 1D, we would also expect to see a transition at approximately 2.6 M urea, the point at which the trimer and monomer populations are equal, but we see no discontinuity in our data for Figure 1B at that point. From our data, it does appear that the hexamer undergoes successive dissociations: from hexamer to two trimers and then the trimer to three monomers. We cannot exclude the possibility of dissociation of the hexamer to six monomers, but it seems relatively unlikely based upon the large amount of trimer present.

Temperature Dependence of the Oligomeric Structures. Using our spectral assignments, we were able to break down our phycocyanin populations into the different oligomeric forms as a function of temperature, Figure 4. Over the limited temperature range that we examined, the hexamer was most stable at approximately 304 K in the absence of urea, but the temperature of maximum stability decreased as the concentration of urea increased. For example, the hexamer

population was a maximum at 297 K in 1.0 M urea and around 288 K at 2.00 M urea. The stability of the other oligomeric forms is more difficult to describe. The trimer population was most stable at temperatures around or below 278 K in 0 M urea, but the temperature with the greatest trimer population increased to approximately 284 K with 1.0 M urea, behavior opposite that of the hexamer. This temperature dependence on the trimer and hexamer populations has been previously observed (*24*). The population of monomer is smallest at the intermediate temperatures studied, and it increases with temperature changes in both directions. Phycocyanin, like many oligomeric proteins, dissociates at both higher and lower temperatures (*32*).

Thermodynamics of the Dissociation Reactions. From the concentrations of the oligomeric forms, the equilibrium constants were calculated as a function of both temperature and denaturant concentration. The averaged equilibrium constants for the hexamer and trimer reactions are shown in Figures 5 and 6; the equilibrium constants increase with greater denaturant concentration and temperature. Although the equilibrium constants we obtained have some small dependence on the preparation of the protein (as seen with the error bars on our data), they consistently displayed linear behavior with denaturant concentration. This trend holds for both the hexamer dissociation and the trimer dissociation above 1.5 M urea at all temperatures studied. The trend also appears to be independent of the denaturant since we also obtain the linear dependence with guanidine hydrochloride for the hexamer dissociation. This linear trend may not hold over the entire concentration range of the urea, but the range of urea concentrations examined does contain the major changes in oligomeric populations.

The equilibrium constant for a dissociation reaction should be independent of protein concentration, as seen with the trimer dissociation for 4.0 and 6.0 μM phycocyanin. We have extensive data on two protein concentrations for the hexamer dissociation, but the equilibrium constants do not coincide especially well, even taking into consideration the error bars on the data. The simplest explanation for this discrepancy is that our samples were not equilibrated at the time of measurement. From all the controls that we have run using absorption spectroscopy, we have confidence that we allowed sufficient time for the sample equilibration, as shown in the Supporting Information.

We have two simple hypotheses that could explain our discrepancy on the hexamer dissociation. First, absorption spectroscopy could be inadequate for measuring the true equilibrium situation of the entire protein complex. We are only probing the tetrapyrrole chromophores along with their local environment. Structural changes to the protein could be occurring on a longer time scale than the changes that occur with the chromophore and its immediate surroundings. A second possible explanation is the preparation dependent variation in equilibrium constants that we have already noted. The data on the hexamer dissociation with 2.0 and 4.0 μM phycocyanin were taken with different protein preparations. We are currently in the process of testing both hypotheses.

As expected from the linear relationship between the equilibrium constant and denaturant, we obtain an exponential relationship between the Gibbs free energy and the denaturant, Figures 5B and 6B. It is hard to discern if this relationship does hold for the trimer dissociation below 1.5 M urea since the signal to noise of the data is poor due to the low population of monomer. This result was unexpected; we expected a linear relationship between ΔG and the denaturant concentration.

The Gibbs free energy of protein unfolding is commonly found through the linear extrapolation method (*10*−*14*) in which the relationship between the Gibbs free energy of the unfolding in urea, $\Delta G^{\circ}_{\text{urea}}$, is related to the Gibbs free energy in the absence of urea, $\Delta G^{0}_{\text{water}}$, with eq 2. The thermodynamics underpinning the linear approximation was developed by Schellman for loss of tertiary structure (*33*). Schellman explicitly excluded interactions between proteins in his derivation; he worked at the limit where [protein] $\rightarrow$ 0. Although this method was explicitly derived for the loss of tertiary structure, other research laboratories have found that dissociation of dimeric proteins appear to be described well using the linear extrapolation model (*10*, *11*, *34*−*36*). In most of the cases described in the literature, the dissociation of a dimer appears to be simultaneous with the unfolding of the individual subunits. We have a relatively unique situation here; the hexamers and trimers dissociate with no discernible loss of the tertiary structure. Generally, the linear approximation is used with relatively high concentrations of denaturant, but we are examining a situation in which the urea induces structural changes at less than 0.5 M concentration. We may be seeing an effect that could be different than what occurs under the more concentrated denaturant concentrations.

$$\Delta G^{\circ}_{\text{urea}} = \Delta G^{\circ}_{\text{water}} + A[\text{urea}] \qquad (2)$$

We examined potential models for the urea disruption in regard to our findings. At least two mechanisms for urea denaturation have been proposed in the literature: preferential binding of urea to the protein backbone in competition with water or an indirect disruption of the protein structure by disruption of water hydrogen bonding with more spectral evidence for the preferential binding mechanism (*37*−*41*). As a first approximation, the assumption that the disruption of oligomeric structure works by the same mechanism as tertiary structure disruption would appear valid.

The mechanism for an indirect disruption of protein structure via disruption of hydrogen bonding in water could be plausible for our hexamer dissociation data. Changes to the bulk solvent with increasing urea concentration could be described by the addition of activity coefficients to the equilibrium expression, but it has been shown that the molar activity coefficient for urea is close to one at the concentrations used (*42*). We are examining the dissociation as a function of pH to determine if the dielectric of the solution is important in our system. The exponential free energies that we observe with our system could be an artifact of our method for monitoring the dissociation reaction. We are using a spectroscopic probe that samples a small volume of the protein and could be insensitive to changes that occur outside the chromophore binding pocket. Thus, we could be missing the presence of significant intermediates in the dissociation reaction or have misidentified an intermediate state as a final state. Recently, deviations from the linear extrapolation method for protein folding have been reported in which the aberrant behavior has been ascribed to changes to the native protein prior to denaturation (*43*, *44*). A single probe of quaternary structure may miss subtle changes in the initial

and final states due to the denaturant. A second method that observes the quaternary structural changes directly could potentially address these issues.

Conclusions. Application of protein unfolding models to the loss of oligomeric structure does not appear to be valid in the case of phycocyanin hexamer and trimers. We find that the equilibrium constants for the dissociation of both structures are linear with denaturant concentration, and that the free energy of the dissociation is exponential with urea concentration rather than linear. The situation examined with phycocyanin is somewhat unusual since the quaternary structure is lost at much lower concentrations of denaturants than normally used, and this may explain the discrepancy that we observe.

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SUPPORTING INFORMATION AVAILABLE

Additional information on the analysis of the absorption by linear deconvolution, the SEC HPLC, and control experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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