Pressure-induced Dissociation and Denaturation of Allophycocyanin at Subzero Temperatures*

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The thermodynamics of assembly of the allophycocyanin hexamer was examined employing hydrostatic pressures in the range of 1 bar to 2.4 kbar and temperatures of 20 to −12 °C, the latter made possible by the decrease of the freezing point of water under pressure. The existence of two processes, dissociation of the hexamer into dimers, \( (\alpha \beta)_3 \rightarrow 3 (\alpha \beta) \), and dissociation of the \( \alpha \beta \) dimers into monomers, \( (\alpha \beta) \rightarrow \alpha + \beta \) have been recognized previously by changes in the absorbance and fluorescence of the tetrapyrrolic chromophores owing to added ligands. The same changes are observed in the absence of ligands at pressures of under 2.4 kbar and temperatures down to −12 °C. On decompression from 2.4 kbar at 0 °C, appreciable hysteresis and a persistent loss of 50% in the absorbance at 653 nm is observed. It results from the conformational drift of the isolated subunits and is reduced to 10% when the highest pressure is limited to 1.6 kbar. The thermodynamic parameters of the reaction \( \alpha + \beta \rightarrow \alpha \beta \) can be determined from pressure effects on perchlorate solutions of allophycocyanin, which consist of dimers alone. Their previous knowledge permits estimation, under suitable hypotheses, of the thermodynamic parameters of the reaction \( 3(\alpha \beta) \rightarrow \{(\alpha \beta)\}_3 \) from the overall pressure effects on the hexamers. Both association reactions have positive enthalpy changes, and the whole hexamer assembly is made possible by the excess entropy.

Allophycocyanin is one of the phycobiliproteins of phycobilisomes, the accessory light-harvesting complexes in cyanobacteria and red algae. Allophycocyanin has been isolated as a trimeric species (Zilinskas et al., 1978; MacColl et al., 1981) of 110 kDa to which we shall refer here as a hexamer. Conversion of \( (\alpha \beta)_3 \) into \( \alpha \beta \) dimers is obtained by the addition of 1 M perchlorate (MacColl et al., 1971; MacColl et al., 1981; Huang et al., 1987) or by lowering the \( \mathrm{pH} \) (MacColl et al., 1980). Dissociation of \( \alpha \beta \) dimers into \( \alpha + \beta \) monomers has been achieved only in the presence of urea at 8 M concentration (Erokhina and Krasnovskii, 1974).

Hydrostatic pressure has been used successfully to explore the thermodynamics and mechanism of assembly of oligomeric proteins. (Silva and Weber, 1993). In this report, we describe how the dissociation of \( (\alpha \beta)_3 \) into \( \alpha \beta \) dimers and then into \( \alpha + \beta \) monomers occurs when the solutions of the protein are subjected to hydrostatic pressures in the range of atmospheric to 3 kbar and temperatures in the range of 10 to −11 °C. The decrease in the freezing point of water under pressure makes it possible to reach a temperature of −20 °C at 2.4 kbar while maintaining the liquid state. In the absence of perchlorate or urea, complete dissociation of allophycocyanin takes place only at these low temperatures, and this has enabled us to show that the process of dimer dissociation by the cold results in a state very similar to that obtained by addition of a classical chemical denaturant, urea, but as a freely displaceable equilibrium. Separation of the two sequential processes of hexamer and dimer dissociation cannot be unequivocally done from the spectral changes under pressure or temperature alone. However the isolation of the pressure effects on the \( \alpha \beta \) dimers by starting with allophycocyanin solutions in 1 M perchlorate, in which the dissociation of the hexamer into dimers is complete, has permitted us to obtain the volume and enthalpy changes of the association of the monomers into dimers. Starting with these data, we have derived the corresponding quantities for the reaction \( (\alpha \beta)_3 \rightarrow 3(\alpha \beta) \) from the overall spectral changes with pressure and temperature, in absence of any added ligands. We thus provide a complete thermodynamic description of the pressure dissociation of a heterohexamer, \( (\alpha \beta)_3 \), into its ultimate subunits, the \( \alpha \) and \( \beta \) monomers.

MATERIALS AND METHODS

The generation and measurement of pressure and the use of spectroscopic methods to determine the degree of dissociation, the changes in volume \( (\Delta V) \) and the changes in the standard free energy of association of the aggregate \( (\Delta G) \) are described elsewhere (Paladini and Weber, 1981a, 1981b; Silva et al., 1986; Weber, 1986). The pressure bomb utilized in this study has been described previously (Paladini and Weber, 1981a). The bomb temperature, measured with a thermocouple, was decreased below room temperature using a methanol circulator and thermostat. Condensation on the bomb windows was avoided by passing a stream of dry nitrogen.

For absorbance measurements, the pressure bomb was placed in the sample compartment of a photodiode array spectrophotometer (3000 Array, of SLM-Aminco). Fluorescence spectra, excited at 600 nm, were determined with a double monochromator spectrofluorometer controlled by software from ISS (Champaign, IL). We recall that change in volume upon reaction, \( \Delta V \), may be obtained by two different procedures (Ruan and Weber, 1989). One involves the change in degree of dissociation with pressure at constant concentration \( (\Delta V_c) \); the other involves the change in pressure required to reach the same degree of dissociation at two different protein concentrations \( (\Delta V_r) \). Unless otherwise stated the values designated here as \( \Delta V \) are exclusively \( \Delta V_c \).

Polarizations of fluorescence were measured and corrected for window birefringence as described by Paladini and Weber (1981a). The sample was excited at 600 nm, and the emission reached the photodetector through an R-59 cut-off filter.

Allophycocyanin from Spirulina platensis was purchased from Sigma, dissolved in 100 mM sodium phosphate buffer (pH 7.5), and stored at 4 °C. Tris-HCl (100 mM, pH 7.5) was employed in all the pressure experiments because of the minimal variation of its pK with pressure (Neumann et al., 1973). Decreasing temperature causes an

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increase in pH of 0.03 pH units/°C. Thus, at −10 °C, an increase in pH of approximately 1 unit with respect to the value at 20 °C is expected.

The αβ dimers were obtained by the addition of 1 M NaClO4 to Tris HCl buffer followed by adjustment of the pH. The purity of the protein was checked by SDS-polyacrylamide gel electrophoresis; only two bands were observed corresponding to the α and β subunits.

The pressure was increased in steps of 200 bar, allowing 15 min for equilibration at each pressure and temperature prior to recording measurements. Control experiments showed that changes in absorbance and fluorescence spectra were completed within 5 min and remained stable for 90 min thereafter.

RESULTS

Spectral Changes Associated with Allophycocyanin Dissociation and Monomer Denaturation—In its hexameric form, native allophycocyanin has absorbance peaks at 620 and 653 nm (MacColl et al., 1981). (Fig. 1). The addition of 1 M perchlorate to the hexameric allophycocyanin results in its dissociation into αβ dimers (MacColl et al., 1980, 1981, 1983), which have an absorbance maximum at 615 nm (Fig. 2). The dissociation of αβ dimers into separate α and β monomers has so far been observed only in the presence of a high concentration of urea (8 M), when the absorbance by the prosthetic group is reduced to about 6% of the native protein. (Erokhina and Krasnoviskii, 1974; Scheer and Weber) (Fig. 2). The 620 nm absorption band of the intact protein (Fig. 1) has been assigned to the chromophores present in the αβ dimers, whereas the 653 nm peak that appears when hexamer is formed has been attributed to exciton coupling between chromophores in adjacent αβ dimers within the hexamer (MacColl et al., 1981; Csatorday et al. 1984; Beck and Sauer, 1992). From the absorption at wavelengths longer than 620 nm, it follows that the integrated dimer absorbance is expected to be 0.27 of that of the hexamer while dissociation into denatured monomers would lower it further to less than 0.06 of the intact aggregate.

In a previous study (MacColl, 1983) it was shown that the hexameric species (αβ)3 of allophycocyanin on excitation of the chromophores at 600 nm exhibits a fluorescence emission with a peak at 662 nm, whereas the emission from the αβ dimers obtained by the addition of 1 M perchlorate has a maximum at 640 nm, with a lower yield. The addition of 8 M urea, which dissociates αβ into α + β, decreases the fluorescence intensity without further spectral shifts. Fig. 3 shows the fluorescence spectra of solutions in neutral buffer (a), 1 M perchlorate solutions (b), at a pressure of 2.4 kbar at 0 °C (c), and in 8 M urea (dashed line). The fluorescence changes follow quantitatively the corresponding absorbance changes.

The limiting fluorescence emission spectrum is very similar to that seen in the presence of urea so that the data of both absorption or fluorescence can be used to monitor the dissociation of hexamer into monomers.

By comparison of Figs. 1 and 2, it is possible to deduce that by the application of pressures of up to 2.4 kbar at 0 °C, the two sequential reactions, (αβ)3 → 3 (αβ) and αβ → α + β occur with the absorbance, and also fluorescence, changes belonging to them running smoothly into each other as dissociation pro-
ceeds. Isolation of the corresponding thermodynamic parameters becomes a matter of fitting the data to a preassigned model. Success in this procedure critically depends on the hypotheses made on the spectral characteristics assigned to the reactions and on the precision of the measurements. To minimize the uncertainties involved, we used a more direct procedure. Allophycocyanin in 1 M perchlorate solution is in the form of dimers, and the thermodynamic parameters for the dimer-monomer equilibrium can be determined by the effect of pressure on these solutions following experience with several other protein dimers (Silva and Weber, 1993). Making use of this data, we can set up the equations corresponding to the two-step dissociation of the hexamers into α and β monomers and derive the thermodynamic parameters of the dissociation of the hexamer into dimers brought about by the application of pressure.

Pressure Dissociation of the Allophycocyanin Dimers—Using perchlorate-treated samples, we were thus able to monitor the effects of hydrostatic pressure and low temperatures upon the dissociation of the αβ dimer without the complications that result from a coupled dissociation of the hexamer. Fig. 2 shows the absorbance spectra of the αβ dimer (1 M perchlorate solutions) as the pressure is stepwise increased to 2.4 kbar at 0 °C. At the latter pressure, the spectrum almost coincides with the spectrum observed when 8 M urea is added to the perchlorate-treated protein at atmospheric pressure and room temperature (22 °C, dashed line). As reported by Chen and Kao (1977) and Chen and Berns (1978), the circular dichroism at 222 nm of biliproteins denatured by urea shows changes parallel to those in the visible absorption spectrum, so that both methods are equivalent as indirect probes of biliprotein unfolding. The observation that application of pressure brings about changes similar to those of urea denaturation indicates that the separated α and β monomers are unstable and that pressure separation is followed by unfolding to an undetermined extent. Therefore the values of the standard changes in free energy ΔG, and volume ΔV, on association, are those for the conversion of unfolded monomers into a folded dimer.

Fig. 4 shows the decrease in the maximal absorbance of the αβ allophycocyanin dimer in 1 M perchlorate as pressure is increased at 0 °C. The decompression pathway (lower curve) exhibits a slight hysteresis. After decompression, the spectral properties of the dimer were fully recovered, indicating complete reversibility of the dissociation reaction at this protein concentration. The application of high pressure (2.4 kbar) at 22 °C promoted a fast dissociation (to γ = 0.47) that reached a plateau in less than 5 min and was practically constant over longer times. Degrees of dimer dissociation (γ) were calculated on the assumption that the plateau values of absorption or emission observed at pressures close to atmospheric pressure correspond to zero dissociation and at highest pressures (or lowest temperatures) correspond to complete dissociation (see for example Figs. 1 and 2). The relation of the degree of dissociation to the dissociation equilibrium constant (K_D) for a dimer is

\[ K_D = 4C_{D} \gamma^{2}/(1 - \gamma) \]  

where \( C_{D} \) is the total concentration of protein expressed as dimer. In terms of the characteristic dimer concentration \( C_{D}^{1/2} \) at which \( \gamma = \frac{1}{2} \), Equation 1 becomes

\[ C_{D}^{1/2}(2C_{D}) = \gamma^{2}/(1 - \gamma) \]  

Fig. 4, inset, shows the plot of \( \ln(\gamma^{2}/(1 - \gamma)) \) against pressure for the data of the figure. The volume change of association (ΔV) and the dissociation constant at 1 bar calculated for the reaction \( \alpha\beta \rightarrow \alpha + \beta \) are summarized in Table I. The volume change found for association of the α and β monomers (104 ml-mol⁻¹) is of the magnitude observed for other dimer-monomer equilibria (Silva and Weber, 1993).

It is also possible to monitor the dissociation and denaturation of allophycocyanin hexamers by measuring the decrease in fluorescence in neutral buffers solution at 0 °C (Fig. 5). The fluorescence at the highest pressure resembles that obtained with 8 M urea, a further indication that the combined effects of pressure and low temperature results in a state of the tetrapyrrolic pigments that mimics that of denaturation at room
temperature by urea. Returning pressure and temperature to initial conditions resulted in recovery of the fluorescence properties of the protein parallel to the changes in absorbance, and the values of $D_V$ and $K_0$ from the fluorescence data are in close agreement with those of absorbance (Table I).

To determine the protein concentration dependence of the pressure effects on the dimer, compression at 0°C was performed at two different protein concentrations. Fig. 6 shows that the pressure curve was uniformly shifted to higher pressures at the higher protein concentration. The pressure difference at $g_{50.5}$, $D_{p1/2}$ is 330 bar, while the theoretical for $D_V = 100 \text{ ml mol}^{-2}$ is $400 \text{ bar}$. The 20% difference of $D_{p1/2}$ between observed and computed values is within the range generally observed in other dimers (Silva and Silveira, 1992; Silva and Weber, 1993).

The dissociation reaction was also examined by varying the temperature at constant pressure (Fig. 7). Starting at atmospheric pressure at 22 °C (solid symbol in Fig. 7, upper left) an increase in pressure to 1.6 kbar produced a very small change in absorbance (filled circles), but a considerably larger one at 2.4 kbar (empty circles). At 2.4 kbar and −11 °C, the final absorption ratio reached was nearly the same as observed by increase in pressure at 0 °C (Fig. 4). At 1.6 kbar, the freezing point is −9 °C, and while the maximum dissociation reached is thus limited, the slope of the change in absorbance with temperature is such as to indicate that the lower limit of absorbance would be reached at a temperature not much different than that at 2.4 kbar.

Effect of hydrostatic pressure on the allophycocyanin hexamer—In the dissociation of the hexamer into dimers, the degree of dissociation $e$ is related to the thermodynamic equilibrium constant by the equation

$$K_0 = 27^eC_H^e(1 - e)$$

(Eq. 3)

where $C_H$ is the concentration of protein expressed as hexamer. In terms of the characteristic concentration $C_{1/2}$ at which $e =$
\[ \frac{1}{2} \text{ Equation 3 reads} \]

\[ \left[ C_{1/2}^\text{H} / 2 C_{1/2}^\text{D} \right]^2 = e^\gamma (1 - \epsilon) \]  

(Eq. 4)

If the constants \( C_{1/2}^\text{H} \) and \( C_{1/2}^\text{D} \), as well as the protein concentration as hexamer, \( C_{1/2} \), are given, Equations 4 and 2 can be coupled by a simple iterative procedure to yield the values of \( \gamma \) and \( \epsilon \) at which, at any fixed conditions of temperature and pressure,

\[ \epsilon C_n = (1 - \gamma) C_0 \]  

(Eq. 5)

In this resolution, the characteristic values of \( C_{1/2}^\text{H} \) and \( C_{1/2}^\text{D} \) at each pressure, \( C_{1/2}^\text{H}(p) \) and \( C_{1/2}^\text{D}(p) \), are related to the corresponding values at atmospheric pressure, \( C_{1/2}^\text{H}(0) \) and \( C_{1/2}^\text{D}(0) \), by the equations

\[ C_{1/2}^\text{H}(p) = C_{1/2}^\text{H}(0) \exp (\mu p V^\text{H} / RT) \]

\[ C_{1/2}^\text{D}(p) = C_{1/2}^\text{D}(0) \exp (\mu p V^\text{D} / RT) \]  

(Eq. 6)

where \( \Delta V^\text{H} \) and \( \Delta V^\text{D} \) are the standard volume changes of the respective association reactions \( 3(\alpha \beta) \rightarrow (\alpha \beta)_3 \) and \( \alpha + \beta \rightarrow \alpha \beta \). The parameters for the dimer monomer equilibrium \( C_{1/2}^\text{D}(0) \) and \( \Delta V^\text{D} \) are known from the observations on perchlorate solutions of allophycocyanin so that the coupling of the sequential equilibria depends upon the choice of the parameters \( C_{1/2}^\text{H}(0) \) and \( \Delta V^\text{H} \). Employment of the independently obtained data on dimer dissociation in perchlorate solutions involves the assumption that the thermodynamic parameters of the dissociation of the dimer in 1 M perchlorate do not materially differ from those that correspond to the dimers generated on dissociation of the hexamers in the absence of perchlorate. We consider this uncertainty preferable to that involved in the simultaneous determination of four unknown parameters instead of two. If we thus employ the two parameters obtained from the observations on 1 M perchlorate solution, we can compute, for any pair of the parameters, \( C_{1/2}^\text{H}(0) \) and \( \Delta V^\text{H} \), the proportions of hexamers, dimers, and monomers at each pressure. As we know (Figs. 2 and 3) that the respective contributions to absorption at 636 nm (see below) or to fluorescence are in the ratios 1.0:2.7:0.05, we can construct the spectral change curve corresponding to the parameters employed, \( C_{1/2}^\text{H}(0) \) and \( \Delta V^\text{H} \), and compare it with the experimental observations. Fig. 1 shows that increasing pressure to 2.4 kbar at 0°C results in a progressive decline in both absorption peaks at 620 and 653 nm but with the decrease in absorption at 620 nm becoming more pronounced than at 653 nm. The 653 nm peak has been assigned to exciton splitting of the absorbance owing to interaction of nearby dimer chromophores within the hexamer, which can be affected by pressure somewhat differently than the overall absorbance. The average absorption across the whole spectrum should be unaffected by the exciton splitting because the exciton splitting is expected to occur with conservation of the oscillator strength. The monotonic decrease in absorption over the whole band can be resolved into contributions from hexamers and dimers individually represented by any specific region of the spectrum; instead we must take the integrated absorption over the whole band as a measure of the hexamer dissociation. The solid line in Fig. 8 corresponds to the unsmoothed average absorption between the limits of 620 and 653 nm. It virtually coincides with the absorption change in the center of mass of the whole band, at 636 nm. This latter set of values was used as characteristic of the changes in hexamer concentration. At 0°C and 2.4 kbar, the absorption throughout the whole band reaches very low limiting values, indicating that for this range of pressures dissociation at 0°C of hexamers into the \( \alpha \) and \( \beta \) monomers is virtually complete. Best fitting of the observed spectral changes obtains for \( K^\text{H} / K^\text{D} = 0.07 \), or \( C_{1/2}^\text{H} = 0.22 \pm 0.06 \) nm and \( \Delta V^\text{H} = 100 \pm 5 \text{ ml mol}^{-1} \). The fractions of hexamers, dimers, and monomers computed for these values of \( C_{1/2}^\text{H}(0) \) and \( \Delta V^\text{H} \), together with their fractional contribution to the absorbance deduced from Figs. 1 and 2 permits calculation of a spectral curve quite close to the experimental points of decrease in absorption at 636 nm at 0°C, as shown in Fig. 9. The difference in the spectral curves that assume dimer contributions of 0.27 (full line) and 0 (broken line) is generally insignificant. The cause is clearly the minimal dimer fraction present at any degree of dissociation, so that we virtually observe a hexamer-monomer equilibrium, in which we are able to separate the constants characteristic of the two stages because of the previous observations on the perchlorate solutions that independently define the parameters \( C_{1/2}^\text{H}(0) \) and \( \Delta V^\text{H} \). We note that the coincidence of the observed and expected absorbance changes does not depend on the exact value of \( C_{1/2}^\text{D}(0) \) so long as \( C_{1/2}^\text{H} C_{1/2}^\text{D} \gg 1 \). This relation between the dissociation constants appears almost inevitable from the smooth changes in absorption (Fig. 1) or fluorescence (Figs. 3 and 5) of solutions of allophycocyanin in buffer compressed at 0°C toward the corresponding spectra observed in 8M urea and the equally smooth changes in absorbance on cooling the solutions below 0°C (Fig. 7). However, we note that the conclusions reached by the agreement of the data of Figs. 1 and 5 with the result of the computations (Fig. 9) substantially depend on specific hypotheses about the molecular origin of the absorption spectrum and on the assumption that the dissociation constant for the dimer-monomer equilibrium in buffer is not significantly smaller than that determined for the dimer in 1 M perchlorate.

Fig. 10 illustrates the hysteresis that occurs on reversal of the changes in absorption with pressure. Though there were considerable differences between compression and decompression associations calculated from the absorbances at both 653 and 620 nm, reassociation on decompression appears to be virtually complete for the 620 nm absorbance but only about 50% complete for the 653 nm absorbance, indicating an imperfect association that does not restore completely the conditions necessary for the recovery of the initial exciton splitting. This imperfect reassociation, which bespeaks a loss of free energy after dissociation, evidently arises from changes in the conformation ("conformational drift") of the separated subunits of oligomeric proteins. It is observed in the dissociation of some dimers by dilution (Xu and Weber, 1980; Silva and Silveira, [55x129]...}
Pressure and Temperature Effects on Allophycocyanin

Effects of compression up to 2.4 kbar followed by decompression to atmospheric pressure at 0°C on the relative absorbances at 653 nm (dark circles) and 620 nm (empty circles) of allophycocyanin in neutral buffer. Dark half-circles, absorbance at 653 nm on decompression from 1600 bar to atmospheric pressure. After decompression, the temperature was raised to 22°C (dashed line). Absorbance ratios equal Abs$_{p=0°C}$/Abs$_{atm;0°C}$, where p is the applied pressure. Protein concentration was 30 µg/ml.

Fig. 9. Spectral curves constructed according to the proportions of hexamers, dimers and monomers calculated with use of the parameters $C_{h}^{D} = 3.2$ nm, $C_{h}^{D} = 0.22$ nm, $\Delta V^h = 104$ ml mol$^{-1}$, $\Delta V^D = 100$ ml mol$^{-1}$. Solid line, absorbance change with pressure for a dimer contribution of 0.27; dashed line, absorbance change for zero dimer contribution. Points, experimental values of the relative absorbance change at 636 nm.

Fluorescence Energy Transfer under Pressure—At 2.4 kbar, lowering the temperature from 22°C, at which most of the protein is associated into hexamers, causes a 5.5-fold increase in fluorescence polarization (Fig. 11). This increase is similar to that observed when allophycocyanin hexamers are converted into $\alpha\beta$ dimers by the addition of perchlorate (MacColl et al., 1980; Canaani et al., 1980; Holzwarth et al., 1990). The low polarization of the hexamer at 22°C, 0.04 is far too small to result from rotational diffusion and was attributed by these authors to the electronic energy transfer among the prosthetic fluorophores in adjacent dimers. The increase of polarization as the $\alpha\beta$ dimer is formed must then reflect the loss of depolar-
ization by energy transfer owing to disappearance of the excitation coupling. In agreement with observations shown in Fig. 10, the fluorescence polarization, like the absorbance at 653 nm, shows a pronounced hysteresis as the temperature is returned to 22 °C.

**DISCUSSION**

Recent studies of the effects of low temperatures on protein folding have shown that supercooling of a single-chain protein in solution causes its denaturation (Sturtevant, 1977; Griko et al., 1988; Privalov, 1990). The studies of protein dissociation by hydrostatic pressure carried out thus far have been limited to temperatures above 0 °C, and the relatively small changes in protein conformation that have been detected in the separated subunits have been considered to be the result of a limited conformational drift, which never achieves the structural reorganization characteristic of protein denaturation (King and Weber, 1986; Silva and Weber, 1993). The decrease in the freezing point of water that occurs under pressure makes it possible to expand the accessible temperature range down to −20 °C at a hydrostatic pressure of 2.4 kbar. Thus one can attain, under equilibrium conditions, the temperatures of the supercooled solutions in which polypeptide denaturation has been observed.

The temperature effects observed at constant pressure show that the association of allophycocyanin is maintained by a large entropy change that opposes a considerably positive enthalpy change, a situation that appears to obtain generally for the association of subunits into oligomers (Lauffer, 1975; Weber, 1993; Silva and Weber, 1993). Allophycocyanin dissociation shows more clearly than other cases the entropic contribution to the protein assembly process, since at room temperature, the state of aggregation of the protein is barely affected by pressure alone. Table I establishes the entropy-driven character of the reversible folding and association of α + β subunits of allophycocyanin into dimers; thus an increase in entropy drives the entire process of association of the unfolded monomers into the complete allophycocyanin hexamer.

Allophycocyanin compression and decompression curves exhibit hysteresis that is more pronounced the greater the extent of dissociation. Hysteresis in the compression-decompression cycle has been observed in most of the oligomers studied (Silva and Weber, 1993). It is attributed to occurrence of a process that follows the separation of the subunits and reduces their affinity for each other. It also results in delayed recovery of the original spectroscopic and enzymic properties of proteins after decompression (King and Weber, 1986; Silva et al. 1986; Ruan and Weber, 1989). As discussed elsewhere (Weber, 1986) this phenomenon appears to be the consequence of the substitution of intersubunit contacts by solvent-subunit contacts. The phenomenon of hysteresis requires that after a reduction in pressure, the degree of dissociation reaches a value that maintains itself for a time, which is at least of the order of the whole compression-decompression cycle (Everett and Smith, 1955; Cooper, 1988; Silva and Weber, 1993). This behavior is well known in the mechanics of solids as characteristic of "materials of fading memory" (Truesdell, 1985), and the observation of the effects of hydrostatic pressure on many oligomers shows that they fall naturally in this category. While materials of fading memory may be expected to share many features of their thermodynamics and dynamics (Gurtin, 1968), proteins are far more heterogeneous than most materials in this category and will inevitably require a theory peculiar to themselves.

Volume changes of association found for hexamer-αβ dimer equilibrium (100 ml/mole) is intermediate between the one found for dimers (60–150 ml/mol) and that for tetratomers (around 220 ml/mol) (Silva and Weber, 1993). Volume changes calculated from data at different temperatures are very similar (Table I), suggesting that the conformational drift of the monomers separated by compression at different temperatures does not influence appreciably the volume change on association, at least in this case.

The observation that the dissociation constants for the hexamer into dimers is approximately 200 times smaller than the dissociation constant of the dimer into monomers in dilute neutral buffer solutions predicates that the former dissociation is inevitably followed by the latter and that the dimer is not an intermediate form that can exist in appreciable concentration in such media, although it is stabilized by 1 M perchlorate. The absence of dimer intermediates has been observed in the dissociation of the tetramers of glyceraldehyde phosphate dehydrogenase (Ruan and Weber, 1989) and glycogen phosphorylase (Ruan and Weber, 1993). Its physiological significance may lie in the need for rapid hydrolytic removal of the protein following the initial stage of dissociation.

The reversibility observed in the reassembly of the αβ dimers after they are converted into α + β subunits by the combined use of pressure and low temperature indicates that inside the cell this step in the assembly process of phycobiliproteins can occur spontaneously. On the other hand, the association into native hexamers may require a particular set of conditions or the presence of cofactors in order to proceed inside the cell, since it is not completely reversible after decompression to atmospheric pressure or increase in temperature, in the dilute buffered solutions studied here. However, the extent of hexamer recovery is proportional to temperature and protein concentration, and inside the cyanobacterial cell the concentration of phycobiliproteins is extremely high. Thus it is also possible that the hexamer assembly proceeds driven by the law of mass action, with some aid from the temperature.

The dissociation-denaturation behavior of allophycocyanin observed in this study is consistent with previous results obtained on intact cyanobacteria (Foguel et al., 1992). With them, the application of high hydrostatic pressure at room temperature resulted in a strong emission with a red shift to 662 nm when the cells were excited with green light. This result suggested that in these conditions, pressure disconnected the phycobilisomes from the thylakoid membranes resulting in the emission of the terminal phycobilisome component, namely allophycocyanin. The emission vanished when a combination of low temperature and high pressure was used, an indication of dissociation and denaturation of the phycobilisomes components. In agreement with those results, we find here that allophycocyanin does not dissociate at room temperature. Dissociation is only observed when pressure is increased at low temperature. Additional studies with intact phycobilisomes and their isolated protein components are in progress in order to obtain further insights into the assembly process.

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Pressure and Temperature Effects on Allophycocyanin

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