Intermediate States of Assembly in the Dissociation of Gastropod Hemocyanin by Hydrostatic Pressure†

Carlos F. S. Bonafe, Jose R. V. Araujo, and Jerson L. Silva*

Departamento de Bioquimica, Instituto de Ciencias Biomedicas, Universidade Federal do Rio de Janeiro, 21941 Rio de Janeiro RJ, Brazil

Received July 19, 1993; Revised Manuscript Received December 8, 1993®

ABSTRACT: Under physiological conditions, the oxygen-transport protein from the gastropod *Megalobulimulus ovatus*, an extracellular hemocyanin, is composed of 20 identical subunits organized into a cylindrical structure (M_r 9 × 10^6; 100 S). It dissociates in the pressure range of 0.4–2.5 kbar, as observed by spectroscopic methods (light scattering and intrinsic fluorescence) and gel filtration. In contrast to what is seen with smaller proteins, especially dimers, the pressure-dissociation curves for hemocyanin show little dependence on concentration, suggesting that native hemocyanin exists as a population of molecules with different free energies of association. The pressure-induced dissociation results from an equilibrium in which each aggregate responds to pressure independently of the others and, at any given pressure, is in one of two states, whole or dissociated, which persists for long times when compared with the duration of the experiment. The subunit–subunit affinity of dissociated hemocyanin is much lower than that of associated subunits, suggesting that a conformational drift of monomers occurs. When hemocyanin undergoes dissociation in the absence of calcium and at high pH (>7.2), a large fraction of the dissociated products changes to a conformation that generates stable intermediate states of assembly, lacking the ability to fully reassemble into decamers and didecamers. These intermediates consist primarily of dimers (M_r 900 000), and they bind oxygen reversibly with a higher affinity than the native hemocyanin. The binding of calcium or protons changes the conformation back to the "associable" state, which finally generates the assembled structure. The dissociation process is highly reversible at low pH (6.8–6.0) or in the presence of millimolar concentrations of calcium. At pH 5.7, dissociation is negligible at pressures up to 2.5 kbar. A decrease in pH from 7.6 to 6.6 increases the half-dissociation pressure ($p_{1/2}$) by 1.3 kbar, corresponding to a stabilization of 1.35 kcal per mole of subunit. The effects of Ca^{2+} and H^+ may mean that, *in vivo*, special ionic conditions or other factors are required to be present at the assembly sites of oligomeric proteins such as hemocyanin.

Oligomeric proteins have a central role in the regulation of biological processes. We now understand well how subunits interact to form small oligomeric proteins such as dimers and tetramers (Jaenicke, 1987; Weber, 1992). However, our knowledge of the thermodynamics and mechanisms of subunit interactions in small oligomers does not lead directly to the understanding of protein–protein interactions in large protein assemblies. As the complexity of a multisubunit assembly increases, the description of its thermodynamic and kinetic properties becomes less tractable. Even the simple transition from dimers to tetramers already leads to changes in these properties, and the changes are accentuated for larger proteins (Silva *et al.*, 1989; Erijman & Weber, 1991).

To explore the thermodynamics and mechanisms of assembly of large multimeric proteins, a method that permits controlled perturbation of the subunit interactions must be employed. Hydrostatic pressure can promote dissociation of oligomeric proteins and viruses (Weber, 1987; Silva & Weber, 1988, 1993). Since pressures of up to 3 kbar have insignificant effects on the properties of single-chain proteins (Heremans, 1982; Weber & Drickamer, 1983), pressures within this range provide reliable information on protein–protein interactions. Pressure effects can be monitored by using light scattering (Engelborghs *et al.*, 1976; Silva *et al.*, 1989; Bonafe *et al.*, 1991), by transport methods such as electrophoresis (Paladini *et al.*, 1987), or by fluorescence spectroscopy (Paladini & Weber, 1981; King & Weber, 1986; Silva *et al.*, 1986, 1992; Royer *et al.*, 1986; Pin *et al.*, 1990; Erijman & Weber, 1992).

In the dissociation equilibria of oligomers, distinct effects appear that have no parallel in the dissociation of small molecules. In many dimers and tetramers, a stable but defective reassociated form, or even a complete loss of ability to aggregate, is observed after a cycle of pressure dissociation and decompression (King & Weber, 1986; Silva *et al.*, 1986). In larger protein aggregates and viruses, reassembly becomes even more difficult (Dreyfus *et al.*, 1988; Silva & Weber, 1988; Bonafe *et al.*, 1991). All of these effects can be explained as the result of a "conformational drift" that occurs following dissociation and appears to be the consequence of the substitution of intersubunit contacts by solvent–subunit contacts and the complete or partial reversal of this conformational drift on reassociation (Weber, 1992; Silva & Weber, 1993).

This study describes the pressure-induced dissociation of a giant protein (M_r 9 × 10^6; 100 S), the hemocyanin of the gastropod *Megalobulimulus ovatus*. This oxygen carrier is a copper-containing protein of twenty identical subunits organized into a pair of cylindrical decameric assemblies (Bonaventura & Bonaventura, 1983). The hemocyanins of the gastropods characteristically have eight folded domains per subunit, each domain housing a pair of oxygen-binding copper atoms (Herskovits, 1988). The molluscan hemocyanins occur as decameric (60 S) or didecameric (100 S) assemblies,

† This work was supported by grants from Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) and Financiadora de Estudos e Projetos (FINEP) of Brazil and by a grant from the European Economic Community (CtI*-CT90-0857) to J.L.S. J.L.S. is a John Simon Guggenheim Fellow.

* To whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* February 1, 1994.

0006-2960/94/0433-2651$04.50/0

which can be dissociated into subunits at extremes of pH or in the presence of denaturants (van Holde & Miller, 1982). The study of such proteins provides a model for the assembly of more complex oligomeric structures such as virus particles.

An apparent violation of the mass action law which has been observed in the dissociation of many hemocyanins (Engelborghs & Lontie, 1973; Siezen & van Driel, 1973; van Holde & Miller, 1982; Herskovits & Villanueva, 1986; Herskovits, 1988) has been attributed to heterogeneity of the subunits. In some cases a difference in the amino acid composition of the polypeptide chains has been found, but in others some sort of structural heterogeneity seems to be the best explanation. Siezen and van Driel (1973) and Engelborghs and Lontie (1973) have proposed that molluscan hemocyanin consists of a large variety of associated forms, each having a unique and very sharp pH zone of dissociation.

In this report, we approach the question of the heterogeneity of subunits in hemocyanin by the use of high-pressure perturbation. We have characterized the hydrodynamic properties of the intermediate states of assembly by gel filtration. The decrease in affinity between subunits after they are separated reveals a conformational drift. Calcium and hydrogen ions favor reassembly, suggesting reversal of the conformational drift to restore the native, high-affinity form.

MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade. Distilled water was filtered and deionized through a Millipore water purification system to >10 MΩ resistance. Unless stated otherwise, the experiments were performed at 20 °C in the standard buffer (0.05 M Tris-HCl and 1.0 mM EDTA, pH 7.4). Tris-HCl buffer was selected because the dependence of its pK_a on pressure is small. At 3 kbar the value of pK_a increases by only 0.1 unit (Neuman *et al.*, 1973).

Hemocyanin Preparation. Megalobulimulus ovatus snails were obtained from Rio de Janeiro. The hemolymph was extracted from several specimens by means of a syringe. Low-speed centrifugation was used to remove any particulate matter. An equal volume of 0.4 M acetate buffer, pH 5.3, was added. Crystalline ammonium sulfate was added, and the fraction that precipitated between 30 and 50% (w/v) ammonium sulfate was taken. The precipitate was resuspended in the same buffer and centrifuged at 250000g_{av} for 2 h. The pellet was resuspended in 0.05 M Tris-HCl buffer, pH 7.4, and was spun at 11500g_{av} for 10 min. The supernatant was further spun at 250000g_{av} for 2 h, and the pellet was resuspended as before. Protein concentration was determined spectrophotometrically using the extinction coefficient $E_{278nm}^{1\%}$ = 13.8 (Airoldi, 1983).

Light Scattering and Fluorescence Measurements under Pressure. The high-pressure optical vessel has been described (Paladini & Weber, 1981). Light scattering and fluorescence spectra were recorded on an ISS 200 spectrofluorometer (Champaign, IL). Light scattering at 435 nm was measured at 90° to the incident light.

The degree of dissociation at a given pressure (α_p) was calculated from the light scattering at pressure p (S_p) as described previously (Silva *et al.*, 1989) by

$$\alpha_p = (S_i - S_p)/(S_i - S_f) \quad (1)$$

The terms S_f and S_i are the light scattering intensity for the completely dissociated and associated forms, respectively. According to theory, the light scattering of the particle is proportional to the average molecular weight (M_r).

Fluorescence spectra at pressure p were quantified as the center of spectral mass $\langle \nu_p \rangle$:

$$\langle \nu_p \rangle = \sum \nu_i F_i / \sum F_i \quad (2)$$

Here F_i stands for the fluorescence emitted at wavenumber ν_i, and the summation is carried out over the range of appreciable values of F. The degree of dissociation $\langle \alpha_p \rangle$ is related to $\langle \nu_p \rangle$ by the expression

$$\alpha_p = (1 + Q(\langle \nu_p \rangle - \langle \nu_f \rangle)/(\langle \nu_i \rangle - \langle \nu_p \rangle))^{-1} \quad (3)$$

In this equation, Q is the ratio of the quantum yields of dissociated and associated forms, $\langle \nu_p \rangle$ is the center of spectral mass at pressure p, and $\langle \nu_f \rangle$ and $\langle \nu_i \rangle$ are the centers of mass for dissociated and associated forms. This calculation has been successfully applied in previous studies (Silva *et al.*, 1986, 1989; Royer *et al.*, 1986; Silva & Weber, 1988).

Size-Exclusion High-Performance Liquid Chromatography. High-performance liquid chromatography gel filtration was performed on a Waters Model 6000A system using prepacked SynChropak GPC300 and GPC500 columns of 250 × 4.6 (i.d.) mm, obtained from SynChrom, Inc. (Linden, IN). Typically, the flow rate was 0.3 mL/min. Elution of the sample was monitored by absorbance at 254 nm. The void volume (V_o) of the column was measured with λ phage DNA or blue dextran, and the total volume (V_t) was measured with ADP. Calibration of the columns was carried out as previously described (Silva *et al.*, 1989). The partition coefficient K_d was calculated from the relation $K_d = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume of the protein.

Oxygen Equilibrium. Oxygen binding measurements were carried out spectrophotometrically as described by Bonaventura *et al.* (1974).

RESULTS

Pressure-Induced Dissociation of Hemocyanin. Figure 1A shows the dissociation of hemocyanin as a function of pressure based on light scattering and fluorescence emission center-of-mass measurements. The decrease in light scattering intensity with an increase in pressure from 1 bar to 2.5 kbar (open circles) was typically 15–20-fold after background subtraction. The half-dissociation pressure ($p_{1/2}$) determined from these data was 1.10 ± 0.06 kbar (average ± SD, N = 5). The standard deviations were smaller than the symbols used (<0.01 unit of degree of dissociation derived from light scattering and <0.02 unit of degree of dissociation derived from center of spectral mass). These experiments were highly reproducible for a given hemocyanin preparation, but the standard deviation for $p_{1/2}$ increased to 0.15 kbar when different protein preparations were used. After decompression, the light scattering intensity returned to only 10–15% of its original value (open circle at left in Figure 1A).

The intrinsic fluorescence spectra of hemocyanin were measured as an indicator of changes in the environment of aromatic residues concurrent with dissociation. Relative to the spectrum at atmospheric pressure, the emission spectrum at high pressure exhibits a red shift of 130 cm^{-1} in the center of mass (Figure 1B). This can be attributed to exposure of tryptophan residues to a medium of increased polarity. The quantum yield of tryptophan emission was not pressure sensitive. The degree of dissociation calculated from the red shift (eq 3) is shown in Figure 1A (filled squares). The close agreement between these different optical methods suggests that the spectral changes are caused by dissociation. A slightly higher value for $p_{1/2}$ (1.2 kbar) is obtained from the dissociation

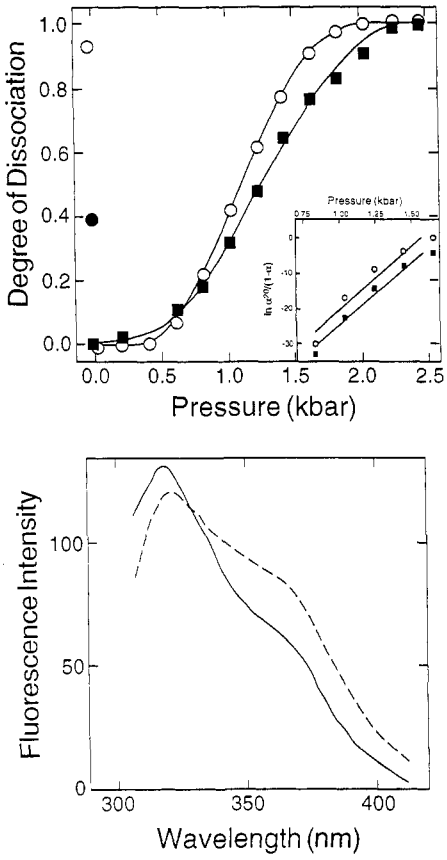

FIGURE 1: (A) Degree of dissociation of hemocyanin vs pressure as determined by light scattering (○) (eq 1) and center of spectral mass of intrinsic fluorescence (■) (eq 3). The data points are means from three determinations. The standard deviations (SD) are smaller than the symbols used. Conditions: hemocyanin, 0.5 mg/mL, in 0.05 M Tris-HCl, pH 7.4, in the presence of 1 mM EDTA was subjected to increasing pressure steps of 0.2 kbar, at a temperature of 20 °C. The symbols at left show the endpoint after return to atmospheric pressure. Inset: Plot of $\ln(\alpha^{20}/(1-\alpha))$ vs pressure based on (○) light scattering and (■) center of spectral mass of intrinsic fluorescence. (B) Fluorescence emission spectra of hemocyanin at atmospheric pressure (—) and at 2.5 kbar (- - -).

curve based on fluorescence. This result may mean that the changes in fluorescence occur mostly at later stages of dissociation, in contrast to light scattering changes. After the return to atmospheric pressure, the fluorescence energy of emission recovered 60% of the value observed for the nonpressurized sample (closed circle, Figure 1A). This degree of reversibility is greater than that determined from light scattering. These results suggest that on decompression the system is trapped in an intermediate state of association. Evidence presented later indicates that this may consist of dimers (H_{c2}). The volume change of dissociation $\Delta V°$ can be calculated from the thermodynamic relation (Weber, 1992; Silva & Weber, 1993)

$$\ln[\alpha_p^n/(1-\alpha_p)] = p\delta V°/RT + \ln(K_{atm}/n^n C^{(n-1)}) \quad (4)$$

where K_{atm} is the dissociation constant at atmospheric pressure, n is the number of subunits, and C is the protein concentration. The slopes of the lines based on light scattering and fluorescence measurements are the same (inset, Figure 1A), such that $\delta V = \Delta V°/n = 43.5 \pm 3.2$ mL/mol of subunit and $\Delta V° = 870$ mL/mol for the whole molecule. If we use $n = 10$, instead of 20, the value of $\Delta V°/n$ does not change significantly because the uncertainty is proportional to $(n-1)/n$ (less than 5%). The more than 15-fold decrease in light scattering suggests that associated hemocyanin dissociates

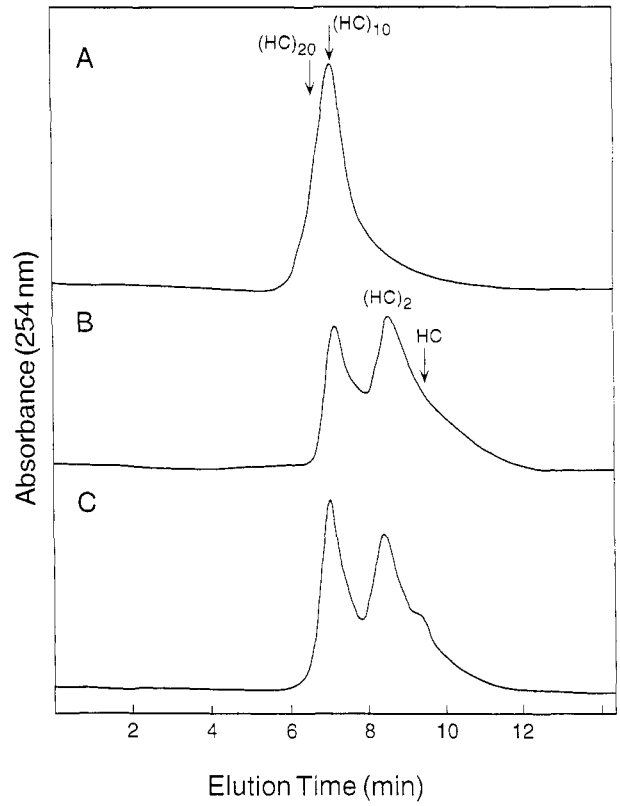

FIGURE 2: Gel filtration analysis of pressure-dissociated hemocyanin (HC). Sizing chromatography (GPC500) of 0.5 mg/mL hemocyanin: (A) control, at atmospheric pressure; (B) subjected to 2.5 kbar for 45 min and injected in the gel filtration column 5 min after decompression; (C) 45 h after decompression. Other conditions were as in Figure 1. Subscripts indicate the number of subunits in the aggregates corresponding to each peak.

into dimers and monomers. The unimodal curve also points out that only a few discrete intermediates seem to exist. Therefore, values of n above 10 are more reasonable.

Analysis of pressurized hemocyanin using high-performance gel filtration chromatography confirmed the results of optical methods. In addition, it allowed us to measure the size and distribution of the different protein aggregates with high precision and in a very short time. The persistence of a dimeric form after the return to atmospheric pressure was revealed. Figure 2 shows a typical elution pattern of hemocyanin from a GPC500 column before and after complete dissociation by pressure (sample subjected to 2.5 kbar and returned to atmospheric pressure). The native form (before pressurization, Figure 2A) elutes as a mixture of decamers (H_{c10}) and didecamers (H_{c20}). In a later experiment (Figure 7), it will be shown that adding calcium or protons shifts the equilibrium toward the completely associated form (H_{c20}). In Figure 2 (without calcium), a small fraction of hemocyanin eluted at 8.5 min corresponding to the dimer (about 900 kDa). Upon return to atmospheric pressure, the hemocyanin eluted predominantly as peaks corresponding to dimers (H_{c2}) and decamers (H_{c10}) (Figure 2B). The elution pattern also shows a shoulder between the two forms H_{c10} and H_{c2}, corresponding to an intermediate aggregate, and an additional shoulder corresponding to the form H_c monomers. The reassociated decamer amounts to about 30% of the sample (Figure 2B). This elution profile remained invariant for several days following pressure dissociation (Figure 2C). In contrast, we observed complete reversibility to decamers if the extent of dissociation was less than 60% (not shown), demonstrating that lack of reversibility is only found at high degrees of dissociation.

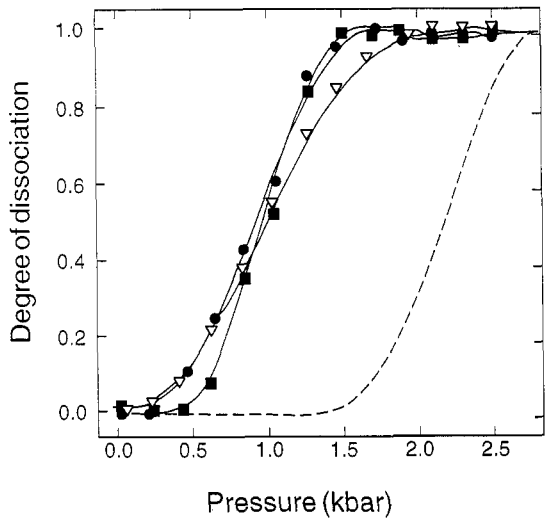

FIGURE 3: Effect of protein concentration on the pressure dissociation of hemocyanin based on light scattering measurements. Plot of degree of dissociation vs pressure at different hemocyanin concentrations: (●) 0.2 mg/mL, (■) 0.5 mg/mL, and (▽) 2.0 mg/mL. The data points are means from three determinations. The standard deviations are smaller than the symbols used. The dashed line depicts the theoretical dissociation curve that would be expected for an increase in protein concentration from 0.2 to 2.0 mg/mL. Other conditions were as in Figure 1.

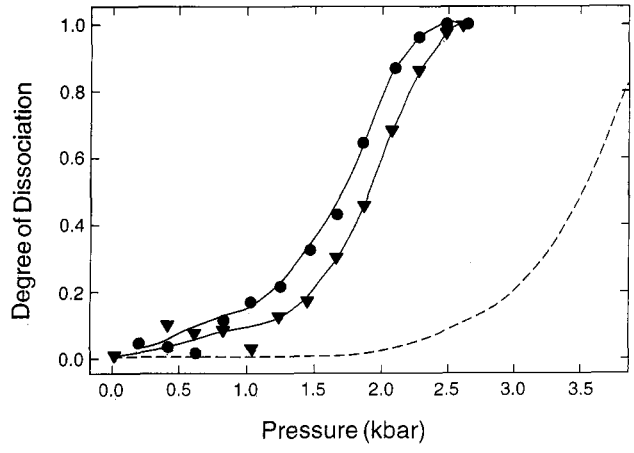

FIGURE 4: Effect of protein concentration on the pressure dissociation of hemocyanin based on light scattering measurements in the presence of 50 mM calcium. Plot of degree of dissociation vs pressure at different hemocyanin concentrations: (●) 0.2 and (▼) 2.0 mg/mL. The data points are means from three determinations. The standard deviations are smaller than the symbols used. The dashed line depicts the theoretical dissociation curve that would be expected for an increase in protein concentration from 0.2 to 2.0 mg/mL. Other conditions were as in Figure 1.

The oxygen binding by native and pressure-treated hemocyanin was measured spetrophotometrically. After decompression, hemocyanin was able to bind oxygen reversibly, suggesting that pressure did not promote denaturation. Hemocyanin subjected to 2.5 kbar for 1 h showed a higher affinity for oxygen (not shown). The value of p_{50} (partial oxygen pressure that promotes binding to 50% of ligand sites) decreased from 2.4 to 1.6 mmHg after pressure treatment. The cooperativity slightly decreased.

Hemocyanin Concentration Effect on the Pressure-Induced Changes. The pressure dissociation measured at equilibrium should depend on concentration (Ruan & Weber, 1989; Silva *et al.*, 1989). Equation 4 permits the calculation of the standard volume change from measurements at a fixed protein concentration, at a series of different pressures, and because of the variable pressure, we designate the volume change ($\Delta V°$) thus obtained as ΔV_p. For a given association–dissociation

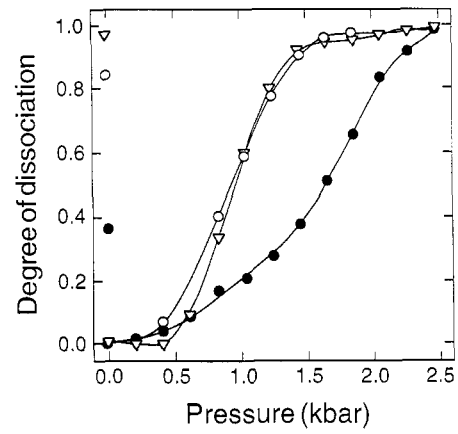

FIGURE 5: Effects of calcium and glycerol on the dissociation of hemocyanin by pressure based on light scattering measurements: (○) control in the absence of calcium or glycerol; (●) in the presence of 50 mM $CaCl_2$; (▽) in the presence of 30% glycerol. The data points are means from two determinations. The standard deviations are smaller than the symbols used. Symbols at left show dissociation after return to atmospheric pressure. Hemocyanin concentration was 0.5 mg/mL. Other conditions were as in Figure 1.

reaction, $H_n \rightleftarrows nH$, a change in protein concentration at a fixed pressure results in a parallel displacement Δp of the plot of ln K_p versus p along the pressure axis. At any fixed value of degree of dissociation, this shift Δp in pressure upon a change in concentration from C_1 to C_2 is given by

$$\Delta p = [(n-1)/n](RT/\delta V_c) \ln(C_2/C_1) \quad (5)$$

where $\delta V_c = \Delta V_c/n$. The subscript c in the terms δV_c and ΔV_c is used to stress the procedure of determining the volume change, that is, by the shift in the pressure dissociation profile with concentration. This relation shows that the smaller the Δp, the larger the δV_c. As shown by eq 5 and as discussed previously (Ruan & Weber, 1989; Silva et al., 1989), any uncertainty in the value of n does not modify significantly the expected value of Δp (see Discussion).

For hemocyanin, the theoretical value of Δp for C_2/C_1 = 10 is 1200 bar. Dissociation of hemocyanin in the concentration range of 0.2–2.0 mg/mL was studied in the absence and in the presence of calcium by observing the decrease in Rayleigh light scattering. No measurable dependence on concentration was observed in the absence of Ca^{2+} (Figure 3). In the presence of calcium (Figure 4), a 10-fold increase in hemocyanin concentration promoted a Δp of 0.2 kbar (SD = 0.04 kbar, N = 5).

Effects of Calcium and Glycerol on the Pressure-Dissociation Profile and on the Reassembly Process. The effect of divalent cations in stabilizing the associated form of hemocyanins is well known (van Holde & Miller, 1985, 1986; Herskovits, 1988). Figure 5 shows that 50 mM calcium chloride promotes an increase in $p_{1/2}$ of 700 bar. The pressure-induced dissociation of hemocyanin in the presence of different concentrations of Ca^{2+} is shown in Figure 6. An appreciable stabilization was observed in 1 mM $CaCl_2$, and in 25 mM $CaCl_2$ the effect was saturated. The free energy of stabilization can be calculated from the following equation (Ruan & Weber, 1988; Bonafe *et al.*, 1991):

$$\Delta\Delta(G_{1/2}) = \Delta p_{1/2}\Delta V° \quad (6)$$

where $\Delta p_{1/2}$ is the difference in half-dissociation pressure for the two conditions and $\Delta V°$ is the volume change of association. In the presence of a saturating concentration of Ca^{2+}, the free energy of stabilization was 1.4 ± 0.2 kcal per mole of

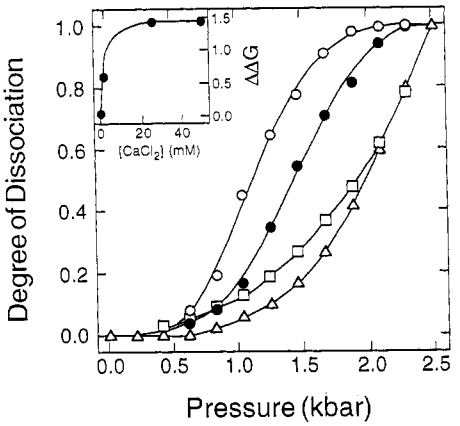

FIGURE 6: Effect of calcium concentration on the dissociation of hemocyanin by pressure based on light scattering measurements: (○) control in the absence of calcium; (●) 1.0 mM $CaCl_2$; (□) 25 mM $CaCl_2$; (△) 50 mM $CaCl_2$. The data points are means from two determinations. The standard deviations are smaller than the symbols used. Other conditions were as in Figure 1, except that the buffer did not contain 1 mM EDTA. Inset: Plot of stabilizing free energy ($\Delta\Delta G$) (eq 6) vs concentration of $CaCl_2$.

associating subunit, adding up to a total of 28 kcal per mole for the whole molecule.

In the presence of 50 mM $CaCl_2$, the light scattering intensity 10 min after a return to atmospheric pressure was equivalent to only 35% dissociation (Figure 5), demonstrating an improved reversibility in the presence of calcium. This observation was confirmed by the HPLC gel filtration data.

The effect of adding calcium to hemocyanin after pressure release is shown in Figure 7. Without calcium, completely dissociated hemocyanin gave rise to a population of intermediate states of assembly that were stable for days (traces B and C in Figure 2). On the other hand, the addition of 50 mM $CaCl_2$ immediately after decompression induced reassociation within minutes. After 50 min, the elution profile was very similar to that of a nonpressurized hemocyanin (Figure 7C).

Figure 8 shows that the dissociation was highly reversible for intermediate degrees of dissociation in the presence of calcium, where there was prompt reassociation to didecameric forms. Even incubation at 2.1 kbar, which causes approximately 80% dissociation, is followed by complete reassociation (Figure 8).

Glycerol is widely used to stabilize different proteins. The effect of adding 30% (v/v) glycerol before the pressure experiment is shown in Figure 5. The pressure-dissociation curve is almost superimposed on the control (without glycerol). Moreover, the increase in light scattering after the return to atmospheric pressure (triangle at upper left) is close to the value obtained in the absence of glycerol (open circle at left). The HPLC gel filtration of the products of dissociation (not shown) revealed a predominant peak at the dimer position (H_{c2}). Reassociation to higher molecular weights was negligible, in contrast to the partial reassociation shown in Figure 2B for recovery of pressure-dissociated hemocyanin in a medium without glycerol.

Hysteresis and Conformational Drift of Pressure-Induced Dissociation. The partial reversibility of hemocyanin dissociation induced by pressure in the presence of calcium allowed us to study the dissociation and reassociation profiles obtained on raising and lowering the pressure through more than one cycle. Figure 9A shows the dissociation and reassociation of hemocyanin in the presence of $CaCl_2$ during two successive cycles of compression and decompression. A pronounced hysteresis is observed, suggesting a significant decrease in

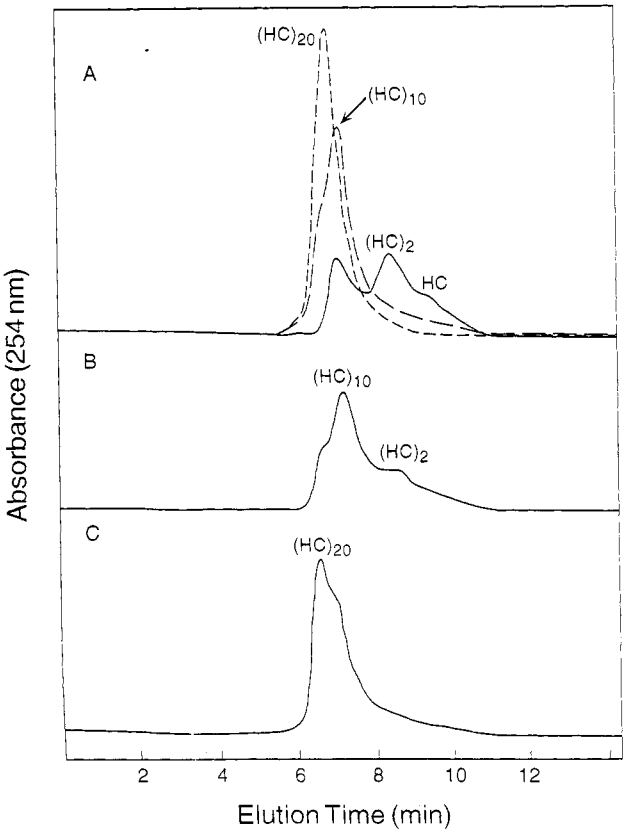

FIGURE 7: Reassembly of pressurized hemocyanin (HC) on adding $CaCl_2$: gel filtration HPLC on a GPC500 column. (A) (– –) control at atmospheric pressure; (- - -) control, at atmospheric pressure in the presence of 50 mM $CaCl_2$; (—) hemocyanin without calcium incubated at 2.5 kbar for 45 min. (B) Hemocyanin pressurized at 2.5 kbar for 45 min and applied to the column 3 min after decompression and addition of 50 mM $CaCl_2$. (C) As in (B) except that the sample was injected into the column 4 h after addition of $CaCl_2$. Other conditions were as in Figure 1.

affinity between subunits once they are separated. A limitation in the interpretation of these data is that our measurement is proportional to the average size and therefore represents different populations of intermediates on the rising curve than on the return. For example, when we analyze the sample that was compressed and decompressed, which gives a degree of dissociation of 0.52, it is constituted of mostly decamers. On the other hand, if the sample is compressed to 1.75 kbar, which produces 50% dissociation, the sample is completely reassociated to didecamers when it is returned to 1 bar (Figure 8).

To determine whether increasing the period of time at each pressure would affect the hysteresis profile, we used different pressurization rates. Figure 9B shows that changing the time at each pressure from 6 to 60 min did not affect the presence of hysteresis. In the latter case, the total duration of the experiment was 24 h. Increasing the time by 1 more order of magnitude is impracticable.

pH Dependence of Pressure-Induced Dissociation, and Reassociation Induced by H^+. The aggregation state of molluscan hemocyanins is dependent on pH, with a tendency to dissociate in moderately acidic and alkaline media (van Holde & Miller, 1982; Herskovits, 1988). The pH effect on the dissociation of hemocyanin by pressure was studied at pH values between 7.6 and 6.6, using light scattering (Figure 10A) and fluorescence (not shown). Both measurements demonstrated a shift of the dissociation curves to higher pressures at lower pH values. Below pH 6.6, there was progressively less dissociation at the highest pressure attained (not shown). At pH 6.6, the degree of dissociation at 2.5 kbar

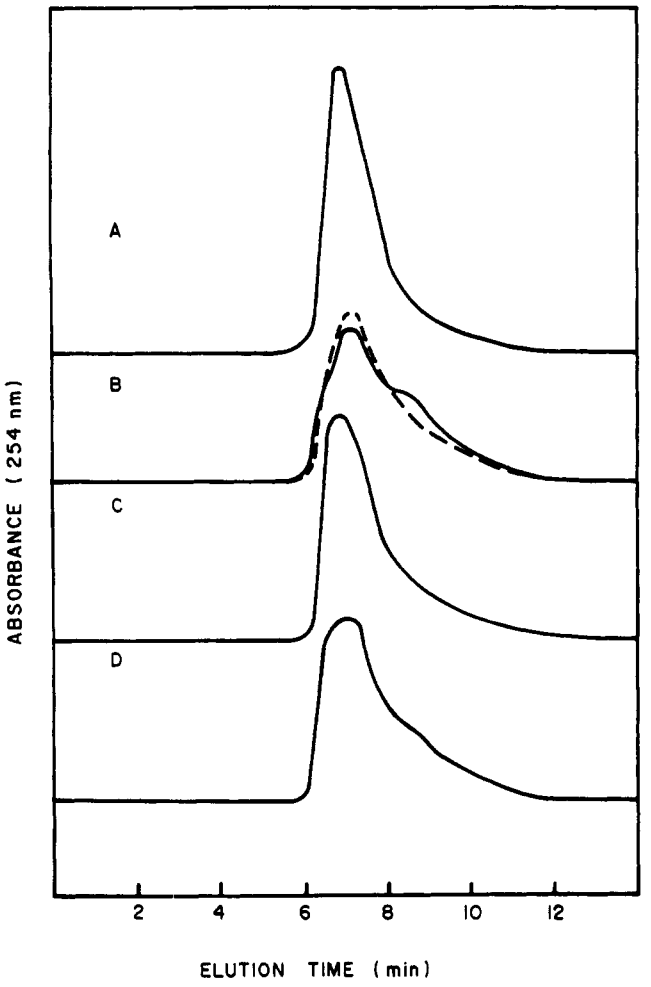

FIGURE 8: Reassembly of pressurized hemocyanin dissociated by different pressures in the presence of $CaCl_2$ (50 mM). (A) Hemocyanin incubated at atmospheric pressure. (B) Hemocyanin incubated at 2.5 kbar for 60 min and then injected in the column 10 min (—) and 2 h (- - -) after return to atmospheric pressure. (C) Hemocyanin incubated at 1.75 kbar for 60 min and then injected in the column 10 min after decompression. (D) Hemocyanin incubated at 2.1 kbar for 60 min and then injected in the column 10 min after decompression.

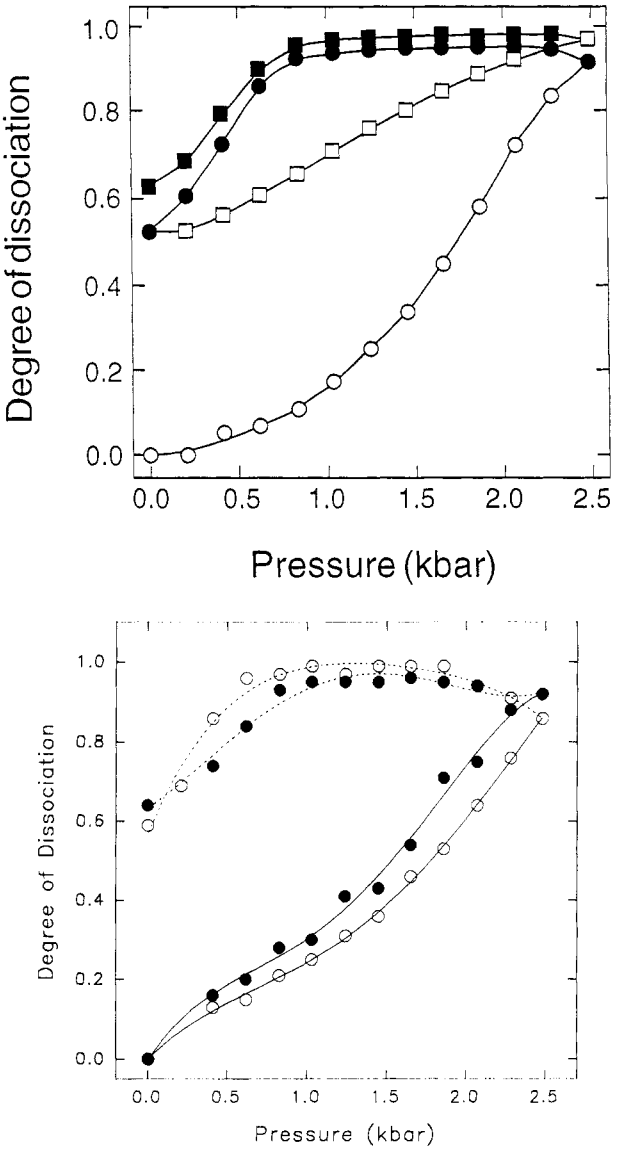

FIGURE 9: (A) Degree of dissociation of hemocyanin vs pressure based on light scattering data in the presence of 100 mM $CaCl_2$ during two successive cycles of compression and decompression. First cycle: (○) compression; (●) decompression. Second cycle: (□) compression; (■) decompression. The data points are means from three determinations. The standard deviations are smaller than the symbols used. Other conditions were as in Figure 1. (B) Degree of dissociation of hemocyanin vs pressure based on light scattering data in the presence of 50 mM $CaCl_2$ during compression (—) and decompression (- - -) at different incubation times at each pressure step: (○) 6 and (●) 60 min. The data points are means from three determinations. The standard deviations are smaller than the symbols used.

did not reach 100%. At pH 5.7, dissociation at 2.5 kbar was negligible: the light scattering intensity differed by less than 5% from the value observed at atmospheric pressure. The data in Figure 10A show a very sharp dependence on pH, with an apparent pK of 7.0–7.2, suggesting the participation of histidine residues (pK_a = 6.5–7.0). Figure 10B shows the logarithmic transformation of the dissociation curves in Figure 10A. The volume change of association (ΔV_p), determined from the slope of the straight lines according to eq 4, did not change with pH. The free energy of stabilization (eq 6) in decreasing the pH by 1 unit (pH 7.6 to 6.6) is 1.35 kcal per mole of subunit. Considering all the subunits in the intact hemocyanin (H_{c20}), the total stabilizing free energy amounts to 27.0 kcal/mol.

High concentrations of protons not only strongly stabilized the associated form but also promoted reassociation of pressure-dissociated hemocyanin. Figure 11 shows the HPLC gel filtration of hemocyanin subjected to 2.5 kbar for 45 min at pH 7.4. After decompression and addition of HCl to reduce the pH to 5.7, reassociation occurred within a few minutes, and after 20 h the reassociation was nearly complete.

DISCUSSION

The pressure-dissociation data on hemocyanin reveal two main findings: (1)The didecamer hemocyanin dissociates into dimers and monomers, and an anomalous dependence on concentration is observed. (2) In the presence of Ca^{2+}, the reassociation occurs through a marked hysteresis. The dissociation is completely reversible depending on the extent of dissociation and on concentrations of Ca^{2+} and H^+, and it becomes progressively less reversible at higher pressures. In the absence of Ca^{2+} and at high pH the dissociated protein appears to be frozen in discrete intermediates: monomers, dimers, and decamers. We discuss these results in the light of the theory of conformational drift and its relation to the appearance of subunit heterogeneity in the monomer–oligomer equilibrium (Weber, 1992; Silva & Weber, 1993).

(1) An Interpretation of the Hysteresis. The theory of conformational drift explains the anomalies found in the dissociation of oligomeric proteins by dilution and by hydrostatic pressure [for reviews, see Weber (1987, 1992) and Silva

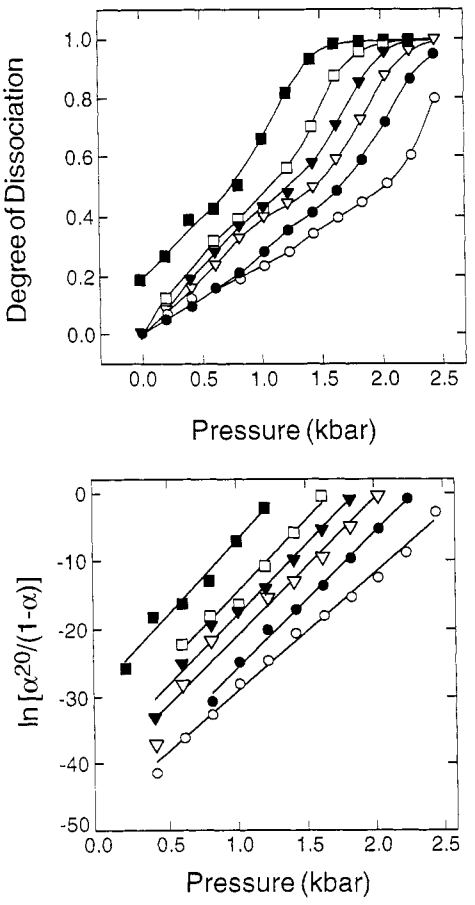

FIGURE 10: (A) Effect of pH on the dissociation of hemocyanin by pressure based on light scattering measurements: 0.5 mg/mL hemocyanin at pH 7.6 (■), 7.4 (□), 7.2 (▼), 7.0 (▽), 6.8 (●), and 6.6 (○). The data points are means from three determinations. The standard deviations are smaller than the symbols used. (B) Plot of $\ln[\alpha^{20}/(1-\alpha)]$ vs pressure: pH 7.6 (■), 7.4 (□), 7.2 (▼), 7.0 (▽), 6.8 (●), and 6.6 (○).

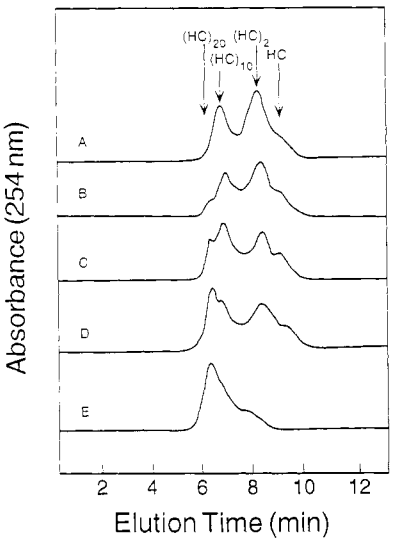

FIGURE 11: Effect of H^+ addition on the reassembly of pressure-dissociated hemocyanin after the return to atmospheric pressure: gel filtration HPLC on a GPC500 column. (A) Hemocyanin, 0.5 mg/mL, dissociated at 2.5 kbar for 45 min at pH 7.4. Immediately after decompression, HCl was added to reduce the pH to 5.5, and the sample was injected in the gel filtration column after (B) 1.5 min, (C) 21 min, (D) 3 h, 15 min, and (E) 20 h, 10 min.

and Weber (1993)]. In the latter case, the main finding, which applies to a number of proteins, is the hysteresis that occurs when a protein dissociated by pressure is decompressed and allowed to reassociate. The hysteresis, which is attributed to a change in conformation (or "drift") that occurs during the period of dissociation, varies from one oligomeric protein to another. Briefly, the theory of conformational drift is based on the fact that the conformation of the subunits is determined by the proximity of the interacting amino acids. Upon dissociation, the contacts between interfacial amino acids are replaced with water, and this directly affects the conformation of the separated peptide chains. In a dynamic equilibrium among associated and dissociated forms, this state of affairs will lead to a time-dependent conformational change after dissociation of the oligomer; this change may be partially or totally reversed after reassociation of the subunits (Weber, 1992; Silva & Weber, 1993).

Cooper (1988) has proposed a slightly different explanation for the conformational drift, one that is still based on the fact that the lifetime of a complex (protein–protein) is relatively short compared to conformational relaxation and energy exchange with the surroundings. Therefore, as Cooper points out, neither free nor complexed protein will attain its true mean equilibrium state, but instead both forms will spend most of their time at some intermediate energy level. Cooper (1988) also addresses the point that the concept of conformational drift does not violate the laws of classical thermodynamics. This is because the multiplicity of accessible conformational states of polypeptides makes these systems completely different from the simpler association of atoms or small molecules. Cooper (1988) successfully simulated the conformational drift through a Langevin procedure. The degree of persistence of a plurality of conformations of the aggregates depends upon the relative rates of the opposing processes of the conformational drift of the free monomers and the regeneration of the most stable forms in the aggregate (King & Weber, 1986; Silva *et al.*, 1986; Weber, 1986; Erijman *et al.*, 1993).

The drift results in the progressive stabilization of the subunit interface in its solvent environment. If the time allowed for this process is sufficiently long, it results, after reassociation, in aggregates with reduced free energies of association. Recovery of the initial conformation after reassociation takes place slowly. In the case of large protein assemblies, such as erythrocruorins (Silva *et al.*, 1989; Bonafe *et al.*, 1991) and the hemocyanin described in this study, restoration of the original aggregate takes a very long time indeed. The hysteresis observed in the presence of calcium during two successive cycles of pressurization and decompression of hemocyanin (Figure 9) suggests a loss of free energy of association when the subunits are separated. Even though the hysteresis can be labeled as a "kinetic" effect, there was only a small difference when the experimental time was increased to 24 h (Figure 9B). The difficulties in regaining the original conformation in large-order oligomers must be even greater than in dimers, because they involve concerted motions of all the contacting surfaces. In a larger multimeric protein complex the difficulties of concerted motions involving a large number of subunits are obvious, and we may surmise that the free energy of association to form the intact aggregate is determined by conditions obtaining at the moment of the initial association. The conformation of each molecular assembly persists through time, giving each particle an individual character. For dimers, the adherence to the law of mass action and the similarity of the times for subunit exchange and dissociation, in contrast to the large difference in these values when tetramers or large aggregates are considered, suggest a simple explanation for the slow rate of regeneration of the most stable forms of the aggregates, and suggest the existence of fractions that differ in their free

Table 1: Analysis of Dependence on Protein Concentration

A. According to the Assigned Value of n in Equation 4

reaction	n	$\Delta V_p/n$ (mL/mol)	$\Delta V_c/n$ (mL/mol)	γ^a	Δp (bar) (expected)
$H_{20} \leftrightarrow 20H$	20	33.0	270	0.122	1620
$H_{20} \leftrightarrow 10H_2$	10	34.5	256	0.135	1460
$H_{10} \leftrightarrow 10H$	10	34.5	256	0.135	1460
	5	37.1	227	0.163	1210
	2	45.1	142	0.317	640

B. According to a Stepwise Dissociation of Hemocyanin

reaction[b] $H_{20} \overset{K_1}{\leftrightarrow} 2H_{10} \overset{K_2}{\leftrightarrow} 10H_2 \overset{K_3}{\leftrightarrow} 20H$	n_{app}	$\Delta V_p/n$ (mL/mol)	$\Delta V_c/n$ (mL/mol)	γ	Δp (bar) (expected)
$K_3 \gg K_2 \gg K_1$	20	33.0	270	0.122	1620
$K_3 > K_2 > K_1$	10–20	33–34.5	>256	<0.135	>1460
$K_3 \approx K_2 > K_1$	10	34.5	256	0.135	1460
$K_3 < K_2 < K_1$	$2 < n < 10$	34.5–45.1	>142	<0.317	>640

[a] $\gamma = \Delta V_p/\Delta V_c$. [b] K_1, K_2, and K_3 are dissociation constants for the three steps in the dissociation and are related to the protein concentration (C) expressed as didecamers: $K_1 = 4C\alpha_1^2/(1-\alpha_1)$; $K_2 = 5^5C^4\alpha_2^5\alpha_1^4/(1-\alpha_2)$; and $K_3 = 4C\alpha_3^2\alpha_2\alpha_1/(1-\alpha_3)$; where α_1, α_2, and α_3 are the fractional dissociation values for the oligomeric forms H_{20}, H_{10}, and H_2, respectively. The experimentally measured α is $\alpha = (2\alpha_1 + 5\alpha_2 + 2\alpha_3)/9$. Calculations were performed assuming different relative values of K_1, K_2, and K_3 in the derived pressure equation: $\ln[(\alpha_1^7\alpha_2^5\alpha_3^2)/(1-\alpha_1)(1-\alpha_2)(1-\alpha_3)] = (\Sigma V)p/RT + \ln[K_1K_2K_3/(16)5^5C^6]$, with $\Sigma V = \Delta V_1 + \Delta V_2 + \Delta V_3$ where ΔV_1, ΔV_2, and ΔV_3 are the association volume changes for steps 1, 2, and 3.

energies of association (Silva & Weber, 1993): They arise from the restricted exchange between those conformations that require displacements of more than two subunits relative to each other.

(2) The Significance of Anomalous Concentration Dependence. Dissociation studies of hemocyanin using salts and alkaline pH values have also shown an anomalous dependence on protein concentration (van Holde & Miller, 1982; Herskovits, 1988). It was proposed (Engelborghs & Lontie, 1973; Siezen & van Driel, 1973) that molluscan hemocyanins consist of a heterogeneous population of different forms, each having a unique and very sharp pH zone of dissociation. Similarly, the anomalous concentration dependence of the pressure-induced dissociation of gastropod hemocyanin might result from an equilibrium in which each particle responds to pressure independently of the others and, at any given pressure, is in one of two states, whole or dissociated, which persists for long times when compared with the duration of the experiment. Each species dissociates at a specific pressure p_i, which is the ratio $p_i = \Delta G_i/\Delta V_i$. For each external pressure p_{ext} applied to the system, there is a split into two populations: the ones with $p_i \leq p_{ext}$ undergo dissociation and contribute to the dissociated fraction (α); the ones with $p_i > p_{ext}$ do not undergo dissociation and represent the associated fraction ($1-\alpha$). The model is simplified by assuming that the different p_i values arise from a distribution of free energies of association (ΔG_i). However, a distribution of volume changes (ΔV_i) or a mixture of both can equally well be proposed.

Equations 4 and 5 show that the volume changes of association can be derived from measurements of changes in dissociation with pressure at constant concentration (ΔV_p) and from changes in dissociating pressure with concentration, at constant degree of dissociation (ΔV_c). Silva and Weber (1993) defined $\gamma = \Delta V_p/\Delta V_c$ as a parameter that can be used to verify experimentally whether protein dissociation by pressure adheres to the law of mass action. In dimers, γ is close to 1 (Paladini & Weber, 1981; Silva et al., 1986, 1992; Ruan & Weber, 1988); in allophycocyanin trimer, it is 0.63 (Foguel & Weber, 1993); in several tetramers $\gamma = 0.25$ and 0.3, and in erythrocruorin and viruses it is below 0.1 [see papers cited in the review by Silva and Weber (1993)]. Ruan and Weber (1989) proposed that the anomalous concentration dependence could be explained by heterogeneity in the free energy of association of the molecular population of the higher oligomers. In the studies described here hemocyanin behaves similarly to erythrocruorin and viruses.

The analysis is simplified in eq 4 by considering the absence of significant amounts of intermediates between aggregate (H_n) and completely dissociated (nH) species. For hemocyanin in the absence of Ca^{2+}, the theoretical value of Δp (eq 5) for $C_2/C_1 = 10$ is 1200 bar. We analyze here the data obtained in the presence of Ca^{2+} because in this condition the changes are more reversible. A 10-fold increase in hemocyanin concentration resulted in a Δp of 200 bar. Considering the reaction $H_{20} \leftrightarrow 20H$, n is 20 and results in a theoretical Δp of 1600 bar. Based on eq 5, we can attribute the disagreement between the observed and the expected values of Δp to differences between the volume changes δV_p (eq 4) and δV_c (eq 5). If we were dealing with atoms or simple molecules, this would not be the case. However, as suggested by Cooper (1984, 1988), proteins are mesoscopic systems, intermediate between microscopic and macroscopic objects, the result being that the conformation of a given protein may fluctuate by many tens of kilocalories per mole. The dynamic character of proteins is generally accepted (McCammon & Harvey, 1988; Karplus & Petsko, 1990). The assembly of "fluctuating" polypeptides into molecular complexes would be expected to increase even further the number of possible states. The discrepancy between the values of δV_p and δV_c probably arises from the permanency of states that differ in their free energies of association.

The value δV_c calculated from eq 5 is 270 mL/mol, about 8 times the value of δV_p (33 mL/mol) from eq 4. The dashed lines in Figures 3 and 4 show the theoretical curves expected for the highest concentration of protein used in these experiments if $\delta V_c = \delta V_p$, as observed for dimeric proteins (Silva *et al.*, 1989, 1992). A question that arises is whether the dependence on protein concentration is affected by the value assigned to n in eq 4 or by the number of steps in the dissociation of a large oligomer. As previously demonstrated (Ruan & Weber, 1989; Silva *et al.*, 1989; Erijman & Weber, 1991), the expected value Δp or δV_c (eq 5) does not vary significantly whether the volume change is the result of two, three, or more steps of the dissociation reaction. Table 1 shows a detailed analysis of the dissociation of hemocyanin and the derived parameters. In Table 1A, different dissociation

orders are considered; in Table 1B, the analysis is based on a stepwise dissociation of hemocyanin, with three microscopic dissociation constants (K_1, K_2, and K_3). Table 1A shows that a change from 2 to 5 in the value assigned to n in eq 4 changes the derived values of $\Delta V_p/n$ from 45.1 to 37.1 mL/mol and the expected Δp from 640 to 1210 bar. When n is increased from 5 to 20, the changes in these parameters are much smaller: $\Delta V_p/n$ reaches 33.0 mL/mol, and the expected Δp reaches 1620 bar. Table 1B shows several models for stepwise dissociation of hemocyanin ($H_{20} \leftrightarrow 2H_{10} \leftrightarrow 10H_2 \leftrightarrow 20H$) and the apparent values of n (n_{app}) depending on relative values of K_1, K_2, and K_3. For all of the models there is a large discrepancy between the expected values of Δp and the experimental values (200 ± 40 bar). The ratio γ is also much less than 1. The discrepancy persists even for the very unlikely case where $n = 2$. These results can only be explained by the existence of a molecular population that is heterogeneous with respect to free energy of association.

The model of heterogeneity has been deeply discussed in a recent review (Silva & Weber, 1993). Differences in the characteristics of the equilibrium with the complexity of the multimeric protein have been demonstrated directly by studies of the exchange of labeled subunits among oligomers in solution. It has been observed (Erijman & Weber, 1991, 1993; Ruan & Weber, 1993) that in tetramers the time required for the dissociation of a fraction of the tetramers by hydrostatic pressure at 0 °C is shorter, by more than 1 order of magnitude, than the subsequent time for exchange of the free subunits and the remaining oligomers. On the other hand, in dimers, the times for dissociation and subunit exchange are very similar, in accordance with the finding of values close to 1 (Silva & Weber, 1993).

The violation of the mass action law is only apparent, and therefore, there is no violation when heterogeneity of the free energies of association is proposed. The model does not avoid the mass action effect. In fact, adherence to the mass law of subpopulations (according to eq 5) is what permits the conclusion that ΔV_c is very large.

(3) Intermediate States of Assembly of Hemocyanin: Effects of Calcium and Protons. Calculations of the degree of dissociation induced by pressure were based on two spectroscopic methods that showed comparable results and agreed with HPLC gel filtration data for the dissociated products. Thus, there appear to be at least three intermediates in the disassembly of the whole molecule (H_{c20}):

$$H_{c20} \rightleftarrows 2H_{c10} \rightleftarrows 10H_{c2} \rightleftarrows 20H_c$$

where H_c is the completely dissociated subunit. A similar scheme has been proposed by Herskovits (1988). The irreversible character of hemocyanin dissociation under some conditions (Figures 1 and 2) might be explained by the conformational drift of the completely or partially dissociated subunits (a $H_c \leftrightarrow H_c^*$ transition).

When the pressure is returned to 1.0 bar, the fraction of the protein that is in the altered conformation associates to form a conformationally altered dimer that does not reassociate to decamer (Figure 2). The hemocyanin dimer might get trapped in this long-lived kinetic intermediate. Calcium, which promotes reassociation, probably interacts with the altered form of dissociated hemocyanin to restore the conformation of the high-affinity form. The existence of an isomerization reaction in the dimer has been previously suggested (Siezen, 1974; van Holde & Miller, 1982). A similar result was found using an annelid hemoglobin (Bonafe *et al.*, 1991) in which calcium promoted reassembly of the dissociated products. The change from pH 7.4 to 5.5 also promotes reassociation of hemocyanin (Figure 11). These observations raise the possibility that the assembly of some oligomeric proteins *in vivo* may require specific ionic conditions. The role of these ionic factors (H^+ or Ca^{2+} in the case of hemocyanin) may be similar to that of certain protein factors that promote the association and renaturation of some proteins, the chaperonins (Ellis & Hemmingsen, 1989; Goloubinoff *et al.*, 1989) or scaffolding protein (Prevelige *et al.*, 1988).

ACKNOWLEDGMENT

We thank Professor Gregorio Weber for helpful suggestions and discussion and for his constant support and encouragement. We also thank Dr. Martha Sorenson and Dr. William Mantulin for critical reading of the manuscript.

REFERENCES

Airoldi, L. P. S. (1983) Ph.D. Dissertation, Universidade Estadual de Campinas, Campinas SP, Brazil.
Bonafe, C. F. S., Villas-Boas, M., Suarez, M. C., & Silva, J. L. (1991) *J. Biol. Chem. 266*, 13210–13216.
Bonaventura, C., & Bonaventura, J. (1983) in *The Mollusca*, Vol. 2, pp 1–50, Academic Press, Inc.
Bonaventura, C., Sullivan, B., Bonaventura, J., & Bourne, S. (1974) *Biochemistry 13*, 4784–4789.
Cooper, A. (1984) *Prog. Biophys. Mol. Biol. 44*, 181–214.
Cooper, A. (1988) *J. Mol. Liq. 39*, 195–206.
Da Poian, A., Oliveira, A., Gaspar, L. P., Silva, J. L., & Weber, G. (1993) *J. Mol. Biol. 231*, 999–1008.
Dreyfus, G., Guimaraes-Motta, H., & Silva, J. L. (1988) *Biochemistry 27*, 6704–6710.
Ellis, R. J., & Hemmingsen, S. M. (1989) *Trends Biochem. Sci. 14*, 339–342.
Engelborghs, Y., & Lontie, R. (1973) *J. Mol. Biol. 77*, 577–587.
Engelborghs, Y., Heremans, K. A., De Maeyer, L., & Hoebeke, J. (1976) *Nature 259*, 686–689.
Erijman, L., & Weber, G. (1991) *Biochemistry 30*, 1595–1599.
Erijman, L., & Weber, G. (1993) *Photochem. Photobiol. 57*, 411–415.
Goloubinoff, P., Gatenby, A., & Lorimer, G. H. (1989) *Nature 337*, 44–47.
Heremans, K. A. H. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 1–21.
Herskovits, T. T. (1988) *Comp. Biochem. Physiol. 91B*, 597–611.
Herskovits, T. T., & Villanueva, G. B. (1986) *Biochemistry 25*, 931–939.
Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117–237.
Karplus, M., & Petsko, G. A. (1990) Nature *347*, 631–639.
King, L., & Weber, G. (1986) *Biochemistry 25*, 3632–3637.
McCammon, J. A., & Harvey, S. S. (1987) *Dynamics of Proteins and Nucleic Acids*, Cambridge University Press, Cambridge.
Neuman, R. C., Kauzmann, W., & Zipp, A. (1973) *J. Phys. Chem. 77*, 2687–2691.
Paladini, A. A., & Weber, G. (1981) *Biochemistry 20*, 2587–2593.
Paladini, A. A., Silva J. L., & Weber, G. (1987) *Anal. Biochem. 161*, 358–364.
Pin, S., Royer, C. A., Gratton, E., Alpert, B., & Weber, G. (1990) *Biochemistry 29*, 9194–9202.
Prevelige, P. E., Jr., Thomas, D., & King, J. (1988) *J. Mol. Biol. 202*, 743–757.
Royer, C. A., Weber, G., Daly, T. J., & Matthews, K. S. (1986) *Biochemistry 25*, 8308–8315.
Ruan, K., & Weber, G. (1988) *Biochemistry 27*, 3295–3301.
Ruan, K., & Weber, G. (1989) *Biochemistry 28*, 2144–2153.
Ruan, K., & Weber, G. (1993) *Biochemistry 32*, 6295–6301.
Siezen, R. J. (1974) *J. Mol. Biol. 90*, 103–113.
Siezen, R. J., & van Driel, R. (1973) *Biochim. Biophys. Acta 295*, 131–139.
Silva, J. L., & Weber, G. (1988) *J. Mol. Biol. 199*, 149–161.

Silva, J. L., & Weber, G. (1993) *Annu. Rev. Phys. Chem. 44*, 89–113.

Silva, J. L., Miles, E. W., & Weber, G. (1986) *Biochemistry 25*, 5780–5786.

Silva, J. L., Villas-Boas, M., Bonafe, C. F. S., & Meirelles, N. C. (1989) *J. Biol. Chem. 264*, 15863–15868.

Silva, J. L., Silveira, C. F., Correia, A., & Pontes, L. (1992) *J. Mol. Biol. 223*, 545–555.

van Holde, K. E., & Miller, K. I. (1982) *Q. Rev. Biophys. 15*, 1–129.

van Holde, K. E., & Miller, K. I. (1985) *Biochemistry 24*, 4577–4582.

van Holde, K. E., & Miller, K. I. (1986) in *Invertebrate Oxygen Carriers* (Linzen, B., Ed.), pp 245–248, Springer, West Berlin.

Weber, G. (1987) in *High Pressure Chemistry and Biochemistry*, NATO Advanced Science Institutes Series C97 (van Eldyk, R., & Jonas, J., Eds.) pp 401–420, Reidel, Dordrecht.

Weber, G. (1992) *Protein Interactions*, Chapman & Hall, New York and London.

Weber, G., & Drickamer, H. G. (1983) *Q. Rev. Biophys. 16*, 89–112.

Xu, G.-J., & Weber, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5268–5271.